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A POLIOMYELITIS VIRUS PATHOGENIC FOR SUCKLING MICE AND MONKEYS

by N. F. STANLEY AND D. C. DORMAN[1]

(From the Institute of Epidemiology and Preventive Medicine, Prince Henry Hospital, Sydney).

(Accepted for publication 1st October, 1952.)

In the course of studies on the epidemiology of poliomyelitis in New South Wales it was found necessary to isolate some of the local strains of virus. As this study also included the isolation of Coxsackie viruses (Stanley *et al.*, 1951), it became a matter of routine to inoculate monkeys, adult mice and suckling mice with material likely to contain the infective agents. By this means numerous strains of Coxsackie virus were isolated as well as strains of poliomyelitis giving the classical reactions in monkeys. This paper describes the isolation and properties of a virus strain (REDWOOD) pathogenic for both monkeys and suckling mice. For the purpose of comparison the properties of a poliomyelitis virus (COBBETT), giving rise to the reactions characteristic of a Brunhilde type, are also included.

MATERIALS AND METHODS.

Cynomolgus monkeys (*M. irus*) were mainly used in these experiments, but *M. mulatta* and *M. nemestrinus* appear to be equally susceptible to New South Wales strains of poliomyelitis when inoculated by the intracerebral route. In each case monkeys were inoculated with 0·25 ml. of material into the left thalamus. The adult and suckling mice used came from the same stock as those referred to in previous papers. Suspensions of tissues infected with virus were made in broth containing penicillin and streptomycin.

Case histories.

COBBETT virus. The patient from whom this strain was isolated was a female aged 20 years who lived in the Sydney metropolitan area. It was reported that the patient became ill six days prior to admission to hospital. Two days before admission neck stiffness was noticeable. This was followed by nausea, fever, headache, pains in back, shortness of breath, a coated tongue and offensive breath. The patient was admitted to hospital on 31/3/51. On 2/4/51 chest movement was nearly absent. Later the same day chest movement was completely absent, there being purely abdominal respiration. The patient's condition deteriorated until there were practically no respiratory movements at all. Some improvement in condition was noted after the patient was placed in a respirator. The following day (3/4/51) the condition was much worse, as evidenced by a distended stomach and the profuse vomiting of black watery fluid. The patient died at 12.45 p.m. The cord only was removed at post-mortem examination. Histological changes compatible with poliomyelitis were observed in the lumbar, thoracic and cervical areas of the cord.

[1] This work was carried out with the aid of the Arthur Browning Bequest.

REDWOOD virus[2]. The patient was a male, aged 3½ years, who lived in the Sydney metropolitan area. A tooth extraction under general anaesthetic was performed on 9/10/50. Nine days later the patient did not feel well and the following night did not sleep. On 20/10/50 the patient was febrile and delirious and complained that it hurt him to lie on his back. The parents noted nystagmus. The child was admitted to hospital on 21/10/50 at 9.30 a.m. in an extremely ill condition, delirious and with irregular nystagmus and twitching of limbs. He showed signs of meningeal irritation and possible weakness of neck and abdomen. There was a condition of spasticity most marked in the arm. This state continued for a few hours with increasing mucus in the throat followed by respiratory depression. A tracheotomy was performed and the patient placed in a respirator. The patient died at 5.30 p.m. on the day of admission. The C.S.F. collected on admission showed 300 leucocytes/cmm., comprising 90 p.c. mononuclear and 10 p.c. polymorphonuclear cells. At post-mortem examination the brain and cord were removed and suitable material placed in fixative or in glycerol-saline.

EXPERIMENTAL.

Isolation and properties of the COBBETT strain.

The histopathological findings of the human cord were in keeping with those of poliomyelitis. Perivascular cuffing was most marked. Specimens of cord taken from the lumbar and cervical regions were ground in cold broth, penicillin and streptomycin were added and the material frozen at —23° C. for three hours. The material was thawed out, cultured for contaminating bacteria and inoculated into animals. Intracerebral and intraperitoneal inoculation of adult and suckling mice yielded no evidence of an infective agent. Intracerebral inoculation of a female monkey (*M. mulatta*) resulted in paralysis of the hind limbs on the fourteenth day. The animal was killed and the brain and cord removed. Histopathological examinations of the cord revealed a picture identical with that of poliomyelitis in monkeys. Infected cord from this passage and the next were sent to U.S.A. in order that the strain could be typed.[3] After five passages this strain shows an average incubation period of four days, following the intracerebral inoculation of a 10 p.c. cord suspension into monkeys. The virus did not produce pocks on the chorio-allantois of chick embryos. A monkey immunized against this COBBETT strain resisted a challenge by the intracerebral route of 1,000 ID_{50} of Brunhilde type virus.[4]

Isolation and properties of the REDWOOD strain.

The brain and cord from the dead child were kept in glycerol-saline at a temperature of —23° C. for seven months before animal inoculations were carried out. A 10 p.c. suspension of brain and cord was made in cold broth, and

[2] We are indebted to Dr. R. Needham, of the Infantile Paralysis Committee for the report on the clinical history and the collection of the brain and cord from this patient.

[3] This was arranged through the courtesy of Dr. H. M. Weaver, Director of Research, The National Foundation for Infantile Paralysis, New York, U.S.A.

[4] Obtained through the courtesy of the George Williams Hooper Foundation, San Francisco, California, U.S.A.

penicillin and streptomycin added. Twenty adult mice and two litters of suckling mice were inoculated by the intracerebral and intraperitoneal routes. The adult mice appeared unaffected. Fourteen suckling mice died on the second and third day after inoculation and showed marked emaciation prior to death. The remaining two mice showed emaciation and retardation of growth, but recovered. The carcases (with viscera removed) of mice dying from this primary infection were prepared by the standard technique and inoculated by the intracerebral and intraperitoneal routes into two other litters. These showed no evidence of being infected and the agent could not be recovered.

The human brain and cord suspension was also inoculated into a monkey (*M. irus*) by the intracerebral route and on to the chorio-allantois of 11-day-old chick embryos. No pocks were produced during five passages. The monkey appeared to be sick after 48 hours and showed right sided paresis for seven days when it appeared drowsy and shivering. The animal was killed eight days after inoculation and the brain and cord removed. Histopathological examination revealed marked perivascular cuffing and concentrations of the glial cells with focal necrosis in the brain, while sections of cord showed perivascular cuffing, focal necrosis and destruction of the anterior horn cells. The brain and cord suspension from this monkey was prepared in the usual fashion and inoculated by the intracerebral route into another monkey (*M. irus*), adult mice, suckling mice and guinea-pigs, by the intraperitoneal route into suckling mice and on to the chorio-allantois of chick embryos. Nothing abnormal was detected except in the monkey and the suckling mice inoculated by the intracerebral route. The suckling mice, after showing marked emaciation and retardation of growth, all died on the second and third day. The infective agent could not be recovered by further passage in suckling mice or suckling rats. The monkey showed paralysis of both hind legs nine days after inoculation of Passage I material. On the following day the monkey was killed and at post-mortem examination the brain and cord were removed. Histological examination revealed a picture in keeping with that of poliomyelitis. A suspension of this monkey brain and cord was inoculated into litters of suckling mice by the intracerebral or the intraperitoneal route. There was no apparent change in mice inoculated by the intraperitoneal route. Those inoculated by the intracerebral route were all dead or markedly emaciated or moribund by the fifth day. The brains of these mice were pooled and a suspension inoculated intracerebrally into a litter of suckling mice and into a monkey (*M. mulatta*). Two of six suckling mice became emaciated and died on the fifth day. The monkey showed paralysis of both hind legs fourteen days after inoculation. Central nervous system material from both monkey and suckling mice caused the characteristic emaciation and death when inoculated by the intracerebral route into further litters of suckling mice. The essential steps of the isolation of the REDWOOD virus and its adaptation to mice and monkeys are given in Fig. 1.

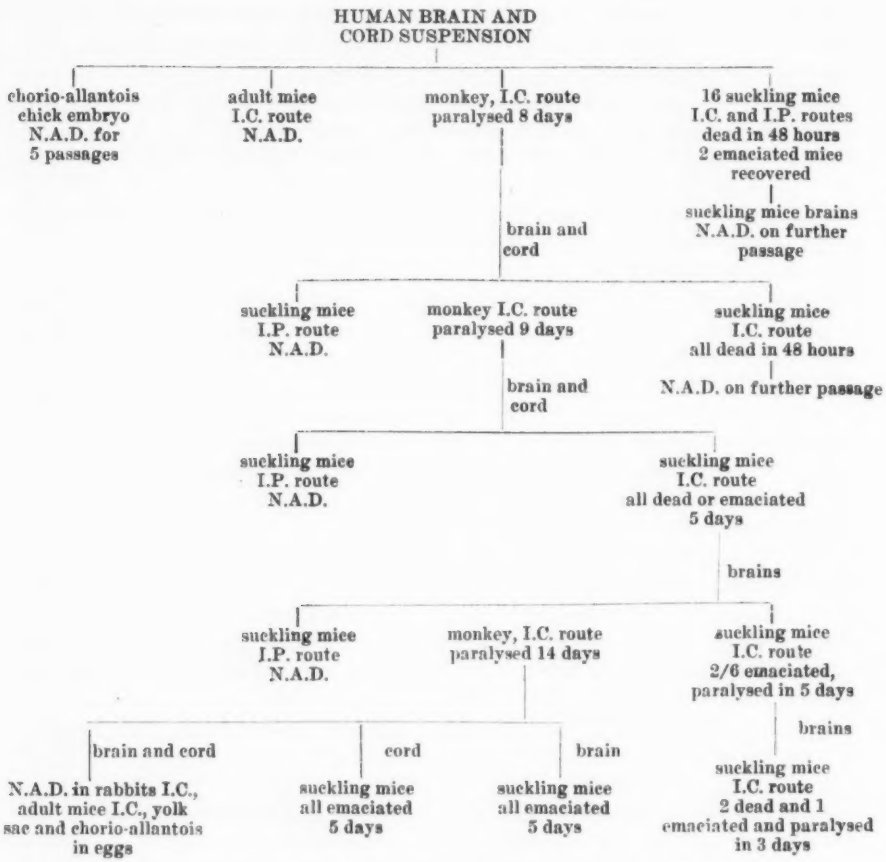
HUMAN BRAIN AND CORD SUSPENSION
chorio-allantois chick embryo N.A.D. for 5 passages
adult mice I.C. route N.A.D.
monkey, I.C. route paralysed 8 days
16 suckling mice I.C. and I.P. routes dead in 48 hours 2 emaciated mice recovered
suckling mice brains N.A.D. on further passage
brain and cord
suckling mice I.P. route N.A.D.
monkey I.C. route paralysed 9 days
suckling mice I.C. route all dead in 48 hours
N.A.D. on further passage
brain and cord
suckling mice I.P. route N.A.D.
suckling mice I.C. route all dead or emaciated 5 days
brains
suckling mice I.P. route N.A.D.
monkey, I.C. route paralysed 14 days
suckling mice I.C. route 2/6 emaciated, paralysed in 5 days
brains
brain and cord
cord
brain
N.A.D. in rabbits I.C., adult mice I.C., yolk sac and chorio-allantois in eggs
suckling mice all emaciated 5 days
suckling mice all emaciated 5 days
suckling mice I.C. route 2 dead and 1 emaciated and paralysed in 3 days

Fig. 1. Diagram showing isolation and early passages for REDWOOD virus.
I.C. = intracerebral inoculation. I.P. = intraperitoneal inoculation. N.A.D. = nothing abnormal detected.

Infection of suckling mice with the REDWOOD virus.

Suckling mice were infected only after intracerebral inoculation. The virus could not be passaged by the intraperitoneal route. The outstanding feature of infection as a result of intracerebral inoculation of mice 24 to 48 hours old was the marked emaciation and retardation of growth which was usually demonstrated between the fourth and eighth day. This was not always followed by death, and the surviving mice did not appear to suffer from muscular weakness when observed for five weeks. Tremors and paralysis were seen in a few

mice, but this was not the common finding. Histological studies on mice, showing emaciation, tremors or paralysis, revealed no pathological changes in the skeletal muscle, cardiac muscle, pancreas, liver or lungs. Sections of cord and brain revealed perivascular cuffing and destruction of horn cells.

Infection of monkeys with REDWOOD and COBBETT strains.

As far as the limited number of monkey inoculations have indicated, the REDWOOD virus behaved in these animals in a manner similar to the classical COBBETT strain of poliomyelitis. Histopathological studies revealed no difference between the two strains. Their pathogenicity for cynomolgus, rhesus and pig-tail monkeys is established although the incubation period for the REDWOOD strain is longer. The temperature charts (Fig. 2) show this difference, which may of course be due to a slower rate of adaptation by the REDWOOD virus.

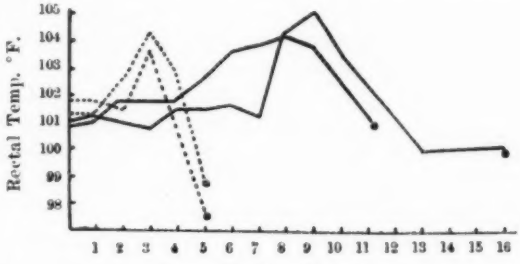

Fig. 2. Rectal temperature of monkeys inoculated by the intracerebral route with REDWOOD or COBBETT viruses.

A dilution of 10^{-4} of a 10 p.c. suspension of COBBETT infected monkey cord caused paralysis of monkeys (*M. nemestrinus*) although the incubation period was prolonged to between seven and ten days. The titration of REDWOOD infected monkey cord has not been carried out.

Comparison with the MEF1 strain of poliomyelitis virus.

Twenty-two suckling mice recovering from infections with the REDWOOD strain were kept for five weeks and inoculated by the intracerebral route with a 10 p.c. suspension of MEF1 infected mouse brain and cord. This strain of MEF1 virus was adapted in this laboratory to suckling mice and was in its sixth passage. All mice became paralyzed or died within fifteen days of inoculation. Furthermore, infection of suckling mice with MEF1 suckling mouse adapted strain produced obviously different symptoms from mice infected with the REDWOOD virus.

DISCUSSION.

The possibility that the REDWOOD strain consists of a mixture of two infective agents, one pathogenic for suckling mice and the other for monkeys, has been considered, especially as this laboratory is carrying out investigations on the Coxsackie group of viruses. The evidence suggests that the possibility of a mixture of a poliomyelitis and a Coxsackie virus is extremely remote. For example, (1) all strains of Coxsackie virus (both myositis and encephalitis types) isolated in this laboratory cause fatal infections in suckling mice when inoculated by the intracerebral, intraperitoneal or subcutaneous routes. REDWOOD virus is pathogenic for these animals *only* by the intracerebral route. (2) The infective material was obtained in the first instance from the brain and cord of a fatal case of poliomyelitis. To our knowledge a fatal case of Coxsackie infection in humans has not yet been described, nor has a Coxsackie virus been isolated from this source. (3) Histopathological observations were in keeping with poliomyelitis. In no instance was myositis observed. (4) The agent was adapted to suckling mice with difficulty and produced a syndrome different from any Coxsackie virus isolated in this laboratory. (5) The infective agent was isolated only in suckling mice and monkeys. It could only be maintained in suckling mice by alternate monkey-suckling mouse passage. The other possibilities to be considered are that the agent is (1) a LEON type of poliomyelitis virus, (2) an atypical Lansing strain, (3) a hitherto undescribed type of poliomyelitis virus, or (4) a virus not related to the poliomyelitis group.

TABLE 1.

Comparison of properties of two viruses.

Properties	Cobbett virus	Redwood virus
Pathogenic for monkeys	+	+
Protects against Brunhilde type poliomyelitis	+	—
Pathogenic for suckling mice	—	+
Pathogenic for adult mice	—	—
Average incubation period (for monkeys)	4 days	10 days
Histopathology characteristic of poliomyelitis	+	+
Relationship to MEF1-Lansing type	None	None

The infection of adult mice by the intracerebral route could not be established. If the virus were of the MEF1-Lansing type, one would expect the virus to become adapted to adult mice. This has not yet been achieved. Furthermore,

mice, recovered from infection with REDWOOD virus, were shown to be equally susceptible to infection with a suckling mouse adapted strain of MEF1 virus, as are normal mice of the same age.

The possibility that the REDWOOD virus is a strain of the LEON type has not yet been investigated. Tissue cultures are being carried out to elucidate this matter further, especially as it has recently been demonstrated that the LEON type of poliomyelitis can be adapted to mice (Li and Habel, 1951). Some of the properties of the REDWOOD and COBBETT strains are summarized in Table 1.

SUMMARY.

The properties of a strain of poliomyelitis isolated from the brain and cord of a child are described. This virus differs from others described in being pathogenic for suckling mice and monkeys but not for adult mice. Its properties are compared with another strain of poliomyelitis recently isolated in this laboratory.

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Stanley, N. F., Hayes, Lila and Dorman, D. C. (1951): Austral. J. exp. Biol., 29, p. 367.

GROUP A COXSACKIE VIRUSES ISOLATED FROM CASES OF POLIOMYELITIS

by N. F. STANLEY AND D. C. DORMAN

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(Accepted for publication 1st October, 1952.)

It was demonstrated that Coxsackie viruses were widespread in Sydney during the summer of 1950-1951, when poliomyelitis was in epidemic proportions (Stanley *et al.*, 1951). It thus became important to study the problems arising from the presence of Coxsackie viruses in the faeces of patients with clinical poliomyelitis. The only methods that have so far been used in this laboratory in an attempt to elucidate these matters have been the isolation of an infective agent from the patient's faeces or throat washings during the acute phase of the disease, and the estimation of the antibody content at suitable periods. Muscle biopsy specimens were not obtained. It is the purpose of this report to describe in detail our findings with three cases, two paralytic and the third non-paralytic, from which poliomyelitis viruses could not be isolated by monkey inoculation.

MATERIALS AND METHODS.

Monkeys used for detecting the presence of poliomyelitis viruses comprised *M. irus*, *M. mulatta* and *M. nemestrinus*. Material for animal inoculation was prepared in the ultra-centrifuge in a manner similar to that described in previous communications (Stanley *et al.*, 1951). The complement-fixation tests (the technique is described in a separate communication) were performed on perspex sheets after the manner described by Kraft and Melnick (1950). The suckling mouse neutralization test has not been modified and was performed in a manner identical with that described in our earlier publication, following the establishment of pure strains for each virus. The faeces and sera from case H124 were supplied through the courtesy of Dr. E. H. Derrick[1].

Case histories.

S112 virus. The patient was a male aged 4 who lived in the country about 40 miles from Sydney. At the onset of the disease on 19/6/51 the following symptoms were noticed—drowsiness, weakness of left arm and leg, pain in the back; the patient was unable to sit up and the speech was slow. The child was admitted to hospital 10 days later. He could not sit up unaided. There was no rash, no adenitis and no apparent loss of sensation. There was slight weakness of the left arm and slight neck stiffness. All reflexes were present and bilaterally equal. A lumbar puncture was performed on 30/6/51. The fluid was clear and under pressure. There were less than 10 leucocytes/cmm.; sugar 65 mg./100 ml., chlorides 690 mg./100 ml., protein 20 mg./100 ml. and globulin not increased. On 4/7/51 there was marked neck rigidity and severe paresis in most muscles of the right leg. A lesser degree of

[1] Deputy-Director, Queensland Institute of Medical Research, Brisbane.

paresis was observed in the deltoid muscle of the right arm. The chief physiotherapist was impressed with the fact that the patient showed marked muscle tenderness and responded very quickly. The patient made an almost complete recovery but is still under observation outside this hospital. Samples of faeces were collected on 3/7/51, throat washings on 2/7/51 and blood on 5/7/51, 22/8/51 and 20/11/51.

H124 virus. The patient was a female aged 17 who was an attendant at a Red Cross Home near Brisbane. Onset commenced on 24/10/50 with a pain in the back and headache. The following day the pain in the back increased and was worse when the head was bent. Loss of appetite, feverishness and shivering also accompanied the backache the next day and the patient was admitted to hospital on 27/10/50. A lumbar puncture performed at this stage revealed a clear and colourless C.S.F. at 100 mm. pressure. There were 40 cells/cmm. comprising 90 p.c. polymorphs and 10 p.c. mononuclears; protein was 50 mg. p.c. The culture was sterile. Penicillin (60,000 units) was given at intervals of 3 hours. The following day the patient appeared flushed and hot and had an increased respiratory rate. The fauces were mildly injected, the tonsils looked chronically infected, and the anterior cervical glands were enlarged. Although the neck was a little stiff there was no evidence of muscular weakness, spasm or tenderness. There was a negative Kernig sign. Temperature was 102·6° F. at 6 p.m. The condition improved during the next few days and the patient was discharged on 3/11/50. A sample of faeces was collected on 2/11/50. Blood was taken on 3/11/50 and 24/11/50.

A145 virus. The patient was a male aged 10 who resided in the Sydney metropolitan area. Onset commenced on 16/1/52 with headache and vomiting. After three days the patient developed weakness of both legs and a stiff neck. The knee-jerk was absent. The calves were tender. The patient, who was drowsy and had a temperature of 101·5° F., was admitted to hospital on 19/1/52. On admission the patient showed some spinal rigidity and could not sit up. On 20/1/52 the knee-jerks were difficult to elicit, the patient could not sit up, but there was no neck stiffness observed. A lumbar puncture was performed, but the C.S.F. was not received by the laboratory until six hours after collection. The specimen was clear and colourless and had less than 10 leucocytes/cmm. The sugar, chlorides, protein and globulin were normal. On 21/1/52, faeces, throat washings and blood were collected for virus examination. Four days later the child still showed weak back muscles and the left tibialis anterior appeared slightly weaker than the right. Cranial nerves were intact. The patient was transferred on 6/2/52 to another hospital and on discharge showed slight weakness of neck muscles. Convalescent serum was collected on 18/2/52. The final diagnosis made was acute anterior poliomyelitis.

EXPERIMENTAL.

Attempts to isolate poliomyelitis viruses.

A virus pathogenic for monkeys could not be demonstrated in the ultracentrifuge deposits from specimens of faeces, although, as will be shown later, Coxsackie virus was present. A cynomolgus and a pig-tail monkey were inoculated by the intracerebral route with the faeces ultracentrifuge deposit from case S112. The rectal temperature chart showed no abnormal fluctuations and neither monkey appeared sick. When challenged two months later with the COBBETT strain of poliomyelitis (Stanley and Dorman, 1953) given by the intracerebral route, both animals developed a pyrexia and became paralysed within 5 days. A similar result was obtained when the ultracentrifuge deposits from the faeces of cases H124 and A145 were given to a cynomolgus and a

rhesus monkey respectively by the intracerebral route. When challenged two months later with the COBBETT strain these monkeys also developed paralysis within five days. It is unlikely, therefore, that a poliomyelitis virus was present in the specimens of faeces collected, although the presence of a LEON type would probably be more ably demonstrated by tissue culture technique (Robbins *et al.*, 1951).

The isolation and identification of the Coxsackie viruses.

The specimens of faeces that failed to yield poliomyelitis virus each contained a Coxsackie strain. After several passages in suckling mice the ID_{50} for brain tissue was 10^{-4} and for skeletal muscle 10^{-6} for both S112 and H124 strains. The ID_{50} for skeletal muscle infected with A145 strain was 10^{-7}. The brain was not titrated. The S112 strain proved to be a serological type not yet reported by us, but 100 ID_{50}'s of the infective agent were neutralized at a dilution of 1/1,280 by hyperimmune mouse serum[2] prepared against Dalldorf group A type 4 Coxsackie virus. This was the first Australian strain of Coxsackie virus to show serological relationship with strains isolated in the U.S.A.

The H124 strain appears to be serologically identical with the W32 type recently described (Stanley *et al.*, 1951). It was demonstrated that this serological type of group A Coxsackie virus has no relationship with Dalldorf group A types 1, 2, 3, 4 or 6. Other sera were not available for testing. A145 strain appears to be a new serological type and is not neutralized by sera obtained from U.S.A. or prepared against any of the six known Australian types. This virus was present in large amount in the faeces and could be isolated from a 1/100 dilution of a 10 p.c. suspension of faeces in broth. The same strain was also detected in the throat washings of the patient collected at the same time as the faeces. The isolation of the virus from throat washings has been extremely rare in this laboratory, but may indicate a heavy infection in this particular case. The three strains produced the destruction of skeletal muscle which characterizes group A strains of Coxsackie virus. Encephalitis and pancreatitis were not observed. A summary of these properties is given in Table 1.

The presence of neutralizing and complement-fixing antibodies to homologous virus in human serum.

The antibody content of the patient's serum was determined by suckling mouse neutralization tests and by complement-fixation tests. In our hands the former method is the more sensitive one. The results of these experiments are recorded in Table 1. Although serum was collected from each individual on admittance to hospital, this cannot always be regarded as acute phase serum. The patient is often not admitted until several days after onset. Our own

[2] This serum was supplied through the courtesy of Dr. Gilbert Dalldorf, Director, Laboratories and Research, New York State Department of Health, Albany, N.Y., U.S.A.

TABLE 1.

Virus	ID_{50} brain	ID_{50} muscle	Serum	No serum	S.M. neutralization tests Serum dilutions 1/20	1/80	1/320	1/1280	Complement fixation tests Serum dilutions 1/5	1/10	1/20	1/40	1/80
S112	10^{-4}	10^{-6}	Dalldorf group A type 4	*8/8	0/6	0/8	0/8	1/8					
			Patient's serum collected after 16 days	8/8	0/6	0/6	2/9	5/8	+	+	+	—	—
			Patient's serum collected after 33 days	8/8	0/6	1/6	7/8	8/8	+	+	—	—	—
			Patient's serum collected after 90 days	8/8	0/8	0/6	8/8	6/6	+	—	—	—	—
H124	10^{-4}	10^{-6}	W32 hyperimmune mouse serum	8/8	0/8	0/8	0/8	1/8					
			Patient's serum collected after 10 days	8/8	0/8	1/8	1/9	8/8	+	+	—	—	—
			Patient's serum collected after 31 days	8/8	0/7	0/7	3/7	8/8	+	+	+	—	—
			Patient's serum collected after 297 days	6/6	1/8	4/8	7/9	6/6	+	—	—	—	—
A145	—	10^{-7}	Patient's serum collected after 5 days	8/8	0/9	2/10	8/8	7/7	—	—	—	—	—
			Patient's serum collected after 33 days	8/8	0/4		4/8	4/4	+	+	—	—	—

* numerator = number of mice paralysed or dead. Denominator = number of mice in litter inoculated. + = fixation of complement.

observations indicate an early rise in antibody titre. The time relationship to onset of disease, isolation of virus and the relative concentrations of neutralizing and complement-fixing antibodies may be seen in Fig. 1.

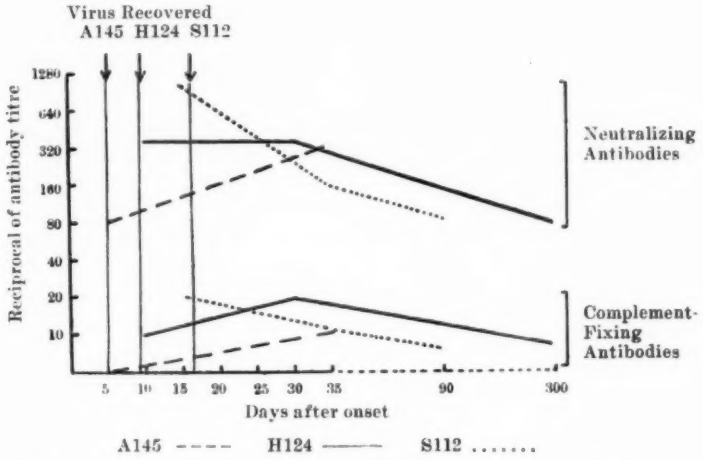

Fig. 1. Neutralizing and complement-fixing antibodies in patients' serum to homologous virus.

The presence of neutralizing antibodies to the MEF1 strain of poliomyelitis.

Antibodies to the MEF1 strain were estimated in the serum collected from patient S112 on 4/7/51 and 22/8/51. To serial double dilutions of serum in broth varying from 1/10 to 1/160 were added equal volumes of a 10 p.c. suspension of infected cord diluted in broth to contain approximately 20 ID_{50} of virus. After standing for 1 hour at room temperature each dilution was inoculated by the intracerebral route into eight adult mice. The results are recorded in Table 2.

TABLE 2.

Neutralization tests with MEF1 poliomyelitis virus against S112 serum.

Eight mice inoculated for each serum dilution.

S112 serum collected on	dilution	Mice dying — Number of days after inoculation														Total No. of mice dying
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
4/7/51	1/20								2	3			1	1	1	8
	1/80				5					1	1				1	8
	1/320			1	1				3	1		1			1	8
28/8/51	1/20				3				2					2		7
	1/80			1	2				3	1	1					8
	1/320			2	1		2		1	1	1					8
No serum				2	1				1	3					1	8

DISCUSSION.

These three cases were presented to illustrate some of the difficulties encountered in interpreting findings associated with Coxsackie and poliomyelitis investigations. Although two patients were diagnosed as paralytic poliomyelitis (S112, A145) and the third as a case of non-paralytic poliomyelitis, no infective agent could be demonstrated by the intracerebral inoculation of the ultracentrifuge deposits of faeces into monkeys. It is realized that to rely solely on monkey inoculation for the demonstration of poliomyelitis viruses may be responsible for the failure to demonstrate LEON types of poliomyelitis. The tissue culture techniques described by Robbins *et al.* (1951) are therefore being used. As far as the isolation of Coxsackie and poliomyelitis viruses is concerned these findings and others reported by us (Stanley *et al.*, in press) are dissimilar to those of Melnick *et al.* (1951) who reported the isolation of both poliomyelitis viruses (by monkey inoculation) and Coxsackie viruses from a large number of cases in an outbreak of poliomyelitis in Easton, Pennsylvania, U.S.A. It is believed at the moment that this dissimilarity may be due to differences in the types of poliomyelitis predominating during the epidemic. Until the presence or absence of LEON type poliomyelitis viruses has been determined this work is difficult to interpret.

It is naturally of great interest to determine whether the Coxsackie viruses, isolated from the faeces and also responsible for the antibody response, bear any relationship to the disease process or the symptoms (especially paralysis) observed at the time of virus isolation. No attempt has been made to isolate either Coxsackie or poliomyelitis viruses from skeletal muscle of patients, and a Coxsackie virus has not been isolated from human brain or cord at post-mortem examination.

In the three cases described in this paper neutralizing antibodies could be detected to a much higher titre than complement-fixing antibodies, although the response followed the same general pattern (see Fig. 1). In some cases the titre of neutralizing antibodies was almost as high as hyperimmune mouse serum. In the case of H124 both neutralizing and complement-fixing antibodies were detected 300 days after onset. Serum was not collected from other patients for such length of time.

SUMMARY.

An account is given of three cases of poliomyelitis, two paralytic, the other non-paralytic, from which Coxsackie viruses of faecal origin were isolated. The patients developed antibodies to the homologous virus to high titre. One strain is related to Dalldorf group A type 4, one to a previously described Australian type (W32) and the third represents a new serological type. Poliomyelitis viruses could not be isolated.

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ANTIBODIES TO COXSACKIE VIRUSES IN POOLED HUMAN SERUM

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(Accepted for publication 1st October, 1952.)

In the course of investigations on the incidence of Coxsackie infections in New South Wales, many different types of virus were isolated (Stanley *et al.*, 1951; Stanley and Dorman, 1953). It was shown that the Australian strains of Coxsackie virus could be divided into three main groups, A, B and C. Groups A and B comprised a number of serological types, but so far all strains in Group C are serologically homologous. The Group B strains were thought to be responsible for a number of cases of Bornholm disease (Stanley *et al.*, 1953), while the Group C virus was shown to be the etiological agent of an outbreak of encephalitis. The association of Group A strains with poliomyelitis is by no means clear.

It is the purpose of this communication to describe the results obtained for Coxsackie antibody estimations in pooled human serum, using virus strains selected from Groups A, B and C.

MATERIALS AND METHODS.

The samples of pooled human serum (P.H.S.) were obtained through the courtesy of Dr. R. J. Walsh, Director of the Red Cross Blood Transfusion Service, Sydney. P.H.S. collected during the years 1943 and 1946 was not available. The P.H.S. samples had been stored at refrigerator temperature until received from Dr. Walsh. They were then heated at 55° C. for 30 minutes and frozen at —23° C. without the addition of germicides.

Antibodies were estimated by the suckling mouse neutralization test which has been described in other publications (Stanley *et al.*, 1951). Four serological types of group A, and one type each of groups B and C were selected for testing against a constant P.H.S. dilution of 1/20.

RESULTS.

Fig. 1 shows the data obtained with suckling-mouse neutralization tests, using P.H.S. samples from 1941 to 1951. For purposes of clarification the results demonstrated with the four serological types of group A virus have

been averaged and plotted separately from the group B and group C virus figures (see Fig. 2). In addition, Fig. 2 shows the results of neutralization tests performed with the group C virus against P.H.S. samples (code Nos CN24, CN25, CN26, CN27) collected in January, 1952.

SERUM (diluted 1/20)		VIRUS GROUP					
		A				B	C
YEAR	CODE NO.	A-a	A-b	A-c	A-4	B-a	C-1
1941	11		□			□	□
1942	370	□	□	□	□	□	□
1944	P71	□	□	□	◧	◨	□
	99	□	□	□	□		
1945	Q78	□	□	□	■	◧	□
1947		◩	◧	◩	■	◩	□
1948	230	◩	◨	◧	■		
	251	◧	◩	■	◩		
	Y99	◩	■	■	■	■	□
	CD11	◩	■	◧	■		
1949	C028	◩	◩	◩	■	◩	□
	C083	◧	◩	■	◧		
1950		◩	□	◩	◩	◩	□
1951	FS3044	◩	◧	◩	■		
	FS3045	■	◧	■	■	■	◨
	FS3046	◩	■	■	◧		

■ = 100 p.c. protection

□ = 100 p.c. deaths

Fig. 1. Neutralization tests with pooled human sera against Coxsackie viruses.

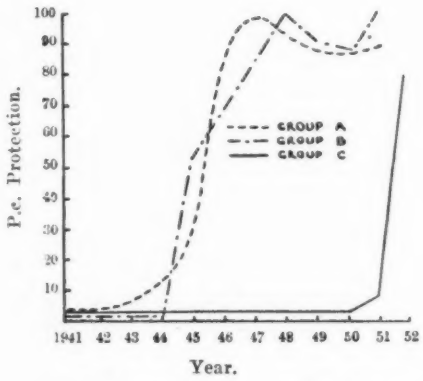

Fig. 2. The presence of neutralizing antibodies to Groups A, B and C Coxsackie viruses in pooled human serum collected 1941-1951.

DISCUSSION.

These preliminary observations revealed that antibodies could not be detected in P.H.S. until 1944 and then only in small amount against two strains of virus. A satisfactory explanation of this finding and of the sudden increase in antibody demonstrated in 1947 with 3 strains and in 1945 with one strain is not yet forthcoming, although investigations are proceeding in an attempt to elucidate the matter. It is felt that if the storage of P.H.S. has resulted in the loss of antibody originally present, then a gradual decline in antibody content would have been revealed as older samples were examined, rather than the sudden absence which has been demonstrated prior to 1944.

An observation worthy of mention has been recorded by Dr. Werner Henle (quoted by Stokes, 1947) with Eastern and Western equine encephalomyelitis viruses. It was found that gamma globulin obtained from donors in the Eastern region of the U.S.A. contained no detectable antibodies against W.E E. virus, although antibodies to E.E.E. virus were present. Stokes (1947) also records the presence of neutralizing antibodies to the Lansing strain of poliomyelitis in gamma globulin prepared from American Red Cross pools.

The sudden rise in antibody concentration to group C viruses, however, may be satisfactorily explained by assuming that the rise is the response to the outbreak of encephalitis which occurred in January, 1952 (Stanley and Dorman, 1953). If this observation is correct, it may then be possible to investigate in retrospect the severity of Coxsackie infections in a community if suitable P.H.S. samples are available. Furthermore, the antibody rise to group C viruses was apparent, although very slight, immediately prior to the group C outbreak. If neutralization tests are carried out promptly, it may thus be possible to predict outbreaks of a similar nature a few weeks before they become clinically apparent.

SUMMARY.

Tests for the presence of neutralizing antibodies to Coxsackie viruses in P.H.S. collected between 1941 and 1951 revealed a rise in concentration to group A and B strains in 1945 and 1946, and a sudden rise to group C strains in 1952. It is suggested that the increase in antibody to group C Coxsackie virus is due to the response evoked by an outbreak of encephalitis in the Sydney metropolitan area early in January.

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STUDIES ON AUSTRALIAN STRAINS OF COXSACKIE VIRUS (GROUPS A AND B)

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The presence of two different groups of Coxsackie virus in Australia has been established (Stanley *et al.*, 1951; Stanley and Dorman, 1953). Because of the small number of cases studied, conclusions of little value could be drawn from these early observations. It is the purpose of this paper to report the isolation and properties of further strains of Coxsackie viruses, the results of antibody estimations on patients' serum and the association of group B virus infections with Bornholm disease.

MATERIALS AND METHODS.

Technique of isolation of virus.

Ultracentrifuge deposits (UCD) from faeces and throat washings were prepared as described in previous communications and the remaining bacterial flora inactivated with a penicillin-streptomycin mixture. The UCD were stored at —23° C. until animal inoculation could be carried out. As a routine, suckling mice were inoculated with UCD by the intraperitoneal route. When monkeys and adult mice were used they were inoculated by the intracerebral route. When evidence existed that an infective agent for suckling mice had been isolated, suitable material from early passages was inoculated into adult mice by the intracerebral route. Suckling mice were observed daily for fourteen days and adult mice daily for twenty-one days. Infant mice were not pooled and redistributed to mothers in numbers of eight, as is the practice in some laboratories. As all material for animal inoculation was proven sterile by culture, and as the infective agents were filtrable, the viral nature of the infective agent was assumed.

The preparation of "pure strains".

Because of the likelihood of isolating a mixture of Coxsackie viruses from faeces, sewage or throat washings, it was deemed advisable to eliminate, if possible, any infective agent of low titre. The routine procedure adopted in this laboratory has been to titrate suitable tissues infected with primary or Passage I virus at dilutions of from 10^{-1} to 10^{-7} and, occasionally, higher to determine the infective dose 50 (ID_{50}) of the virus strains. Following this procedure, mice, becoming paralysed or showing evidence of infection above or at a dilution corresponding to the ID_{50}, were selected for a further titration and the process repeated. Mice infected with a dilution of virus above the ID_{50} in the second titration were then selected to provide material for stock virus, for neutralization tests and for complement-fixing antigens. In this way it was hoped to lessen the possibility of working with mixed virus preparations so that more value could be placed on the evidence of overlapping antigens between virus strains.

Suckling-mouse neutralization tests.

Neutralization tests were not carried out until pure strains were available and the ID_{50} had been calculated according to the method of Reed and Muench (1938). The technique of the test, using approximately 100 ID_{50} of virus, did not differ from that previously described (Stanley *et al.*, 1951). All sera were inactivated at 55° C. for 30 minutes and stored at —23° C. without preservative.

EXPERIMENTAL.

Patients selected for examination.

Faeces, throat washings and two specimens of blood were collected from patients admitted to hospital with a provisional diagnosis of poliomyelitis, non-paralytic poliomyelitis, encephalitis, Bornholm disease, aseptic meningitis or myositis. The number of patients examined in this series was 100, together with a control series of 20 patients in one of the surgical wards of this hospital. Attempts to isolate poliomyelitis viruses were made in selected cases by the intracerebral inoculation of monkeys (*M. mulatta*, *M. irus* or *M. nemestrinus*). It is realized that not all strains of poliomyelitis may be isolated by this method.

The 100 cases studied in this series were admitted to hospital between September, 1950, and December, 1951. This time covers a period when the monthly incidence of poliomyelitis in New South Wales was high (8/100,000 population in January, 1951) and also a period of low incidence (1/100,000 population from August, 1951, to December, 1951). The final diagnoses made by the clinician without knowledge of this laboratory's findings were as follows:

Acute anterior poliomyelitis	54 cases
Bornholm disease	18 cases
Myositis	6 cases
Abortive poliomyelitis	5 cases
Aseptic meningitis	3 cases
Pneumonia	3 cases
Encephalitis	2 cases
Influenza	2 cases

and one case each of mumps, lumbago, osteomyelitis, tonsilitis, infective hepatitis, sinusitis and polioencephalitis.

The nature of the agents isolated.

During the period under discussion, 16 agents, infective for suckling mice, were isolated. These comprised 2 strains of herpes, 3 strains of Coxsackie virus (group B) and 11 strains of Coxsackie virus (group A). Three of the group A strains were isolated from samples of raw sewage; one strain was isolated from the control series of twenty patients and one strain from material supplied from another State. The results are summarized in Table 1.

TABLE 1.

Case No.	Final Diagnosis	Nature of agent	Source	Serological type
W32	Bornholm Disease	Coxsackie group A	faeces	Au
J33	A.A.P.*	Coxsackie group A	faeces	Aa
H124	Aseptic meningitis	Coxsackie group A	faeces	Aa
J37	A.A.P.	Coxsackie group A	faeces	Ab
B58	Myositis	Coxsackie group A	throat	Ab
S112	A.A.P.	Coxsackie group A	faeces	A4
S119	A.A.P.	Coxsackie group A	throat	Ac
A145	A.A.P.	Coxsackie group A	faeces	Ad
LN3	(control case, N.A.D.)	Coxsackie group A	faeces	—
E46	Mumps	Coxsackie group B	faeces	Ba
T80	Bornholm disease	Coxsackie group B	faeces	Ba
M222†	Bornholm disease	Coxsackie group B	faeces	B3
P29	A.A.P.	herpes	throat	
B48	A.A.P.	herpes	throat	

* Acute Anterior Poliomyelitis.

† This strain was isolated by Dr. E. H. Derrick, of the Queensland Institute for Medical Research.

The classification of the Australian Coxsackie strains according to groups and serological types.

Groups.

The separation of the strains into two groups was made mainly on histological grounds, but other differences between the groups also served to distinguish them. One of these was the clinical syndrome of infection in suckling mice. The group B strains were markedly different from the group A strains. An experienced observer could, in most cases, predict the group correctly when a strain was first isolated. When suckling mice were inoculated by the intraperitoneal route, the incubation period for group B strains was longer than that for group A strains. Mice infected with a group B virus as a rule showed the following features, listed in order of frequency: emaciation, wrist-drop, tremors (especially extended and rapidly moving tail), cyanosis and prolonged time before death once signs of infection were noticeable. The mice might become moribund, but rarely showed paralysis of hind limbs. Occasionally, after a long incubation period in mice, group B strains produced an effect which deserved to be recorded. The mice were usually about 12 to 20 days old when this effect was observed. The hind limbs only were affected; the toes were unable to touch the ground. As most of the mice could still move rapidly, they walked on their heels with the toes turned out.

The syndrome produced by infection with the group A strains has been described elsewhere and by other workers. Mice became paralysed and moribund. This is rapidly followed by death.

The group B viruses proved to be more labile than the group A strains which are resistant to many germicides (Stanley *et al.*, 1951). Both group A

and group B strains could infect 5-day old mice, but these were not as susceptible to infection as younger mice.

A further difference noted between the groups was the concentration of the virus in the tissues. The ID_{50} for infected muscle suspensions of group B strains was often considerably lower than for group A viruses, the difference in titre being many thousand-fold.

Histological examination revealed that all group A strains caused the extensive destruction of skeletal muscle that is characteristic of this group. A description of the myositis with photomicrographs has been published elsewhere (Stanley *et al.*, 1951). All group B strains produced marked inflammatory changes in the central nervous system. These consisted of perivascular cuffing, focal necrosis, pyknosis and diffuse encephalitis. Meningitis was marked in some preparations. Destruction of the pancreatic acini was not observed. Focal myositis was observed with all the group B strains isolated, but the destruction of skeletal muscle was not as obvious as that seen with group A infections.

Serological types.

Two methods were used to investigate the antigenic structure of the Coxsackie viruses isolated. They were the suckling-mouse neutralization test and the complement-fixation test. Before either test was carried out, pure strains were establishd for each virus. The results of the complement-fixation tests are reported elsewhere, but data obtained with the neutralization tests are recorded in this paper.

Group A strains. Of the twelve strains isolated ten were examined. Strains S5 and S6 were isolated from raw sewage and strain LN3 from the control series. The ten strains appeared to fall into five antigenic types (see Table 2), only one of which was related to those found in the U.S.A. The strain concerned is S112, which is neutralized by serum prepared against group A type 4 virus.[1] Because the opportunity has not yet presented itself to check our strains with the entire range of serological types recently isolated in the U.S.A., and because new serological types are being readily demonstrated both overseas and in Australia, we have decided to retain the Australian nomenclature for those types not yet proven to be similar to those occurring elsewhere. Where an Australian type has been shown to be identical with an American one, the nomenclature adopted by Dalldorf is used. With group A strains we have W32, J33 and H124, belonging to type a (A-a); strains J37, B58 and S4 to type b (A-b); strain S112 to Dalldorf's type 4 (A-4); strain S119 to type c (A-c); and strain A145 to type d (A-d). The group B strains

[1] Sera from viruses of group A types 1, 2, 3, 4 and 6 and group B types 1 and 2 were supplied through the courtesy of Dr. Gilbert Dalldorf, Director, Laboratories and Research, New York State Department of Health, Albany, N.Y., U.S.A.

have been labelled in an identical manner, but have the prefix B. Thus we have B1, B2, B3 types, etc., in the American classification, and B-a, B-b, B-c, etc., in the Australian classification. It is realized, of course, that this is a temporary measure used to facilitate our serological and epidemiological work until such time as the system used by Dalldorf can be adopted in this country.

It has been our custom to test the pure strain of a new virus against the different hyperimmune mouse sera at dilutions of 1/20. If neutralization occurs, then the test is repeated with additional dilutions of sera up to 1/1,280. If no neutralization occurs it is assumed that serological relationship cannot be demonstrated by this method. This is the reason for recording some of the results, seen in Table 2, at serum dilutions of only 1/20. On the other hand, when neutralization is known to occur, the lower serum dilutions may be omitted in the repetitive neutralization tests. Table 2 shows the results of neutralization tests between group A strains and some hyperimmune mouse sera.

TABLE 2.

Suckling-mouse neutralization tests with group A Coxsackie viruses.

Virus strain	Neutralization titre of serum against							Serological type
	W32	J33	J37	S4	S112	S119	H124	
W32	>1280	>1280	<20	<20			1280	a
J33	>1280	>1280						a
J37	<20		>1280					b
S4	<20		1280	1280				b
B58	<20		1280	1280				b
S112*	<20			<20	1280	<20		4
S119	<20			20	<20	1280		c
H124	1280			<20			1280	a
A145	<20			<20	<20	<20		d

* This strain is neutralized by Dalldorf's group A type 4 serum.

Group B strains. Group B hyperimmune sera against types 1, 2 and 3 were supplied by Dr. Dalldorf. The results of neutralization tests with strains E46, T80 and M222 are recorded in Table 3.

TABLE 3.

Suckling-mouse neutralization tests with Group B viruses.

Virus	Reciprocal of dilutions of serum prepared against Group B									
	Type 1	Type 2	Type 3				Type a			
	20	20	20	80	320	1280	20	80	320	1280
T80	8/8	8/8	8/8				0/8	0/6	0/7	0/8
E46	7/7	6/6					0/6	1/8	2/7	7/7
M222	9/9	12/12	0/10	0/7	0/5	3/6	9/9	6/6		

Numerator = number of suckling mice paralysed or dying.
Denominator = number of suckling mice in litter inoculated.

It may be seen that T80 and E46 are antigenically related and behave similarly in that they are not neutralized by sera prepared against group B types 1, 2 or 3 viruses. On the other hand, the Queensland strain M222 is neutralized by group B type 3 serum to a significant titre (1/1,280).

Antibody response of patients to infection with Coxsackie viruses.

The antibody titre of the sera of patients with group A infections has been studied in detail by Stanley and Dorman (1953), using both the suckling mouse neutralization test and the complement-fixation test to estimate antibody. Readers are referred to this paper for information on S112, H124 and A145 strains. It may be mentioned here that no indication of the presence of infection with a poliomyelitis virus was observed although two of the patients were paralysed.

The antibody response of patients with Bornholm disease against the group B virus isolated from the faeces of some of them during the acute phase is recorded in Fig. 1. The titre of neutralizing antibodies in the patient's serum 10 days after onset of Bornholm disease was, in one case, as high as that recorded for a good hyperimmune mouse serum. It is likely that the T80 strain was responsible for an outbreak of Bornholm disease in the Sydney metropolitan area late in March, 1951 (see case numbers S79, T80 and W81 in Table 4). This same strain is thought to be responsible for an outbreak of Bornholm disease in Tasmania. Pertinent data are recorded in a later section of this paper.

The presence of virus in raw sewage.

In the summers of both 1950 and 1951, samples of raw sewage collected by the Metropolitan Water, Sewerage and Drainage Board were tested for the presence of virus. Coxsackie viruses of group A only were readily isolated from each sample. The two strains (S5 and S6) isolated in 1951 have not yet been typed. Strain S4, isolated in 1950, belongs to the Ab type. Attempts to isolate poliomyelitis viruses by monkey inoculation of the UCD of the same samples of sewage were unsuccessful. It is of interest to mention at this stage that, although several strains of poliomyelitis were readily isolated by monkey inoculation of brain and cord suspensions of fatal cases in this laboratory, no strain of poliomyelitis could be isolated from the faeces of a patient yielding a Coxsackie virus, irrespective of whether the patient became paralysed or not. Group B viruses have not yet been isolated from sewage, and samples of sewage collected during winter months have not yet yielded group A viruses.

Concentration of virus in the tissues of suckling mice.

All mice were inoculated by the intraperitoneal route with suitable infective material. The ID_{50} of 10 p.c. suspensions of the brains or limbs of paralysed mice or mice exhibiting the syndrome characteristic of infection with

group B strains was determined with pure strains of each virus. With group A strains the concentration of virus in muscle was higher than that in brain. With group B strains the reverse was usually the case. Furthermore, the ID_{50} for group A strains for muscle usually exceeded that of group B strains by about 1,000-fold. The average of many titrations showed that for skeletal muscle the ID_{50} for group A strains was 10^{-7} and for group B strains $10^{-3.5}$. The ID_{50} of brain tissue for both groups was 10^{-5}.

VIRUS	CASE NO.	SERUM COLLECTED DAYS AFTER ONSET	RECIPROCAL OF SERUM DILUTIONS 20	80	320	1280	STATE
T80	T80	5	■	■	■	◪	N.S.W.
"	"	10	■	■	■	■	
"	"	186	■	■	■	◪	
"	S79	18	■	■	◪	⊘	
"	W81	10	■	■	■	⊘	
"	T62	7	□	□	⊘	⊘	
"	S63	7	□	□	⊘	⊘	
"	O66	13	□	□	⊘	⊘	
"	B189	2	◪	□	□	□	Tasmania
"	"	15	■	■	◪	◪	
M222	M222	4	□	□	□	⊘	Q'land
"	"	88	■	■	◪	⊘	

■ = 100 p.c. protection
□ = 100 p.c. deaths
⊘ = test not done

Fig. 1. Antibody response of patients, diagnosed as Bornholm disease, to Group B Coxsackie viruses.

CASE NUMBER	SERUM COLLECTED	RECIPROCAL OF SERUM DILUTIONS 20	80	320
0187	14/3/52	■	◪	◪
	25/3/52	■	■	■
B189	13/3/52	■	□	□
	25/3/52	■	■	◪
H190	14/3/52	■	■	■
	25/3/52	■	■	■
S192	14/3/52	■	■	■
H193	14/3/52	■	◪	■
C194	16/3/52	□	□	□
	25/3/52	■	◪	■
0196	19/3/52	■	◪	□
H197	17/3/52	■	■	■
	25/3/52	■	■	◪

□ = 100 p.c. deaths
■ = 100 p.c. protection

Fig. 2. The antibody response to Coxsackie Group B type a (strain T80) in the sera of patients from Scottsdale, Tasmania, during an outbreak of Bornholm disease. Six patients, showing no significant antibody response, are omitted.

Control cases.

At a time when the 1950-51 epidemic of poliomyelitis in New South Wales was at its highest incidence, 20 cases were selected from the surgical wards in this hospital for examination for the presence of Coxsackie viruses. One strain

(LN3) was isolated from a faecal specimen. This was shown to be a group A type b virus. It is realized from our observations as well as those of Huebner *et al.* (1951) that Coxsackie viruses may be very widespread and that such a small number of cases does not constitute an adequate control. Further studies are now being carried out on the natural occurrence of these infective agents with respect to seasonal distribution and other factors. It is worthy of note that in only two cases was the presence of a Coxsackie virus demonstrated in the UCD of throat washings, a finding inconsistent with that of Huebner *et al.* (1951).

Evidence of Coxsackie infection in Australian States other than New South Wales.

Queensland. Through the courtesy of Dr. E. H. Derrick of the Queensland Institute of Medical Research, material was forwarded for examination for Coxsackie viruses as well as sera for the detection of antibodies. Only one group A strain (H124) was isolated and some of its properties were described in a separate communication (Stanley and Dorman, 1953). The H124 strain fell into serological type Aa.

The strain M222 was isolated by suckling mouse inoculation in Queensland and infected brains were sent to this laboratory for study. It was shown to be a group B type 3 virus (see Table 3), and the antibody response of the patient from whom it was recovered is given in Fig. 1. The sera of other Queensland patients with similar clinical findings neutralized this group B type 3 strain to a titre which was pathognostic of a recent infection.

Tasmania. The Government Medical Officer, Dr. K. M. Benn, of Scottsdale, Tasmania, directed our attention to an outbreak of Bornholm disease occurring in this town. Through his courtesy, acute and convalescent samples of sera, throat washings and faeces were collected and mailed to this laboratory. Although Coxsackie viruses could not be demonstrated in these specimens, neutralizing antibodies to Coxsackie group B strain T80 were demonstrated in the sera of a number of patients. The characteristic rise in antibody to high titre was considered to be evidence of recent infection with a Coxsackie strain, similar antigenically to T80 (see Fig. 2). A report on this outbreak will appear elsewhere (Benn, in preparation).

DISCUSSION.

In studying the properties of Coxsackie viruses isolated in Australia it has been our practice to obtain "pure strains" by selecting mice infected with virus at dilutions above the ID_{50}. A repetition of this process was thought to lessen the likelihood of studying preparations containing two infective agents. This could be a danger when working with agents from sewage and faeces, and

was realized by Beeman *et al.*, 1951, who isolated two group A strains from the throat of a patient with herpangina. Group A strains have not yet been isolated in this laboratory from patients with herpangina.

The marked difference between group A and group B Coxsackie viruses (histopathology, virus concentration in tissues, antigenic structure, syndrome of infection, etc.) is also reflected in the nature of the human disease associated so far with the Coxsackie viruses. The group B strains appear to behave differently from the group A strains as far as their epidemiology is concerned, and their association with Bornholm disease is rapidly being established in England (Findlay *et al.*, 1950; Brown *et al.*, 1952), in U.S.A. (Lazarus *et al.*, 1952), and in Switzerland (Thelin and Wirth, 1951). Such has proved to be the case in Australia, where the group B strain T80 (not related serologically to types isolated in U.S.A.) has been shown to be the most likely etiological agent of an outbreak of Bornholm disease in Scottsdale, Tasmania, and also of a small outbreak in the Sydney metropolitan area during the year preceding the Tasmanian outbreak.

It is apparent that group A and group B Coxsackie viruses are widespread throughout Australia, as evidenced by their isolation from sewage, the presence of antibodies in pooled human serum (Stanley *et al.*, 1953) and the demonstration of infection with the viruses in Queensland, Tasmania and New South Wales. Although some outbreaks of Bornholm disease may be the result of infection with a group B virus, the isolation of group A viruses from patients clinically diagnosed as acute anterior poliomyelitis is not so easily interpreted (Stanley and Dorman, 1953). The Easton epidemic recently described by Melnick *et al.*, 1951, is of interest because of the isolation of poliomyelitis and group A Coxsackie strains from more than half of the cases selected for examination, and because of the severity of the epidemic. A much closer study of the relation of the clinical course of a disease to the isolation of Coxsackie viruses and the antibody response of patients is clearly indicated. The epidemiological picture may be clarified by a study of the antibody content of pooled human sera and further investigation of the natural occurrence of the agents during winter and summer.

SUMMARY.

Further studies on the occurrence of group A and group B Coxsackie viruses have resulted in the association of group B infections with Bornholm disease in Sydney and Tasmania. The differences between group A and group B viruses have been shown to be marked. Both agents apparently have wide distribution in the more populated areas of Australia. The techniques of isolating and typing the viruses are described. Five serological types of group A virus and one type of group B virus were isolated in Sydney during the 1950-1951 epidemic of poliomyelitis.

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A HITHERTO UNDESCRIBED GROUP OF COXSACKIE VIRUSES ASSOCIATED WITH AN OUTBREAK OF ENCEPHALITIS

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In the course of epidemiological studies with poliomyelitis and Coxsackie viruses in Australia it became evident that in January, 1952, a dramatic increase had occurred in the number of cases of encephalitis admitted to the Prince Henry Hospital and reported elsewhere in the Sydney metropolitan area. After four weeks there was a rapid decline in incidence. It is the purpose of this communication to describe the isolation and properties of a hitherto undescribed infective agent believed to be closely associated with the outbreak.

MATERIALS AND METHODS.

The techniques used for the isolation of Coxsackie and poliomyelitis viruses from the ultracentrifuge deposits of human material were the same as those described in previous communications. Likewise the method for establishing pure strains and performing suckling-mouse neutralization tests was essentially the same except that mice no older than 24 hours had to be used.

EXPERIMENTAL.

The incidence of Coxsackie and poliomyelitis infections in the Sydney metropolitan area from October, 1950, to March, 1952.

The data presented in Fig. 1 show the monthly incidence of poliomyelitis in the Sydney metropolitan area, recorded as the number of cases per 100,000 population. The curve shows two peaks, one in January and one in April. This effect was demonstrable in all age groups in cases from the Sydney metropolitan area, but was not obvious in graphs of the country cases. On the same graph are plotted the number of Coxsackie strains isolated per month and the number of patients selected for examination. The patients examined in this series comprised every individual admitted to hospital with a provisional diagnosis of poliomyelitis, encephalitis, myositis or Bornholm disease. Tests for the presence of virus and of antibodies to infective agents were carried out irrespective of the final diagnosis made. It may be seen that in the summer of 1950 there was an increase in the number of Coxsackie viruses isolated. These were, with one exception, group A strains and comprised a number of serolo-

gical types. At point C on the graph there occurred a sharp increase both in the number of cases examined and in the number of Coxsackie viruses isolated, and at a time when the poliomyelitis incidence was low. It was subsequently found that in nearly every case a final diagnosis of encephalitis was made by the clinician and that the viruses isolated comprised one serological type that differed in many of its properties from Coxsackie viruses of groups A or B, that have previously been described by us (Stanley *et al.*, 1951, 1953a; Stanley and Dorman, 1953). The remainder of the experimental section of this paper will be devoted to studies on the properties of this group of agents provisionally designated Coxsackie group C.

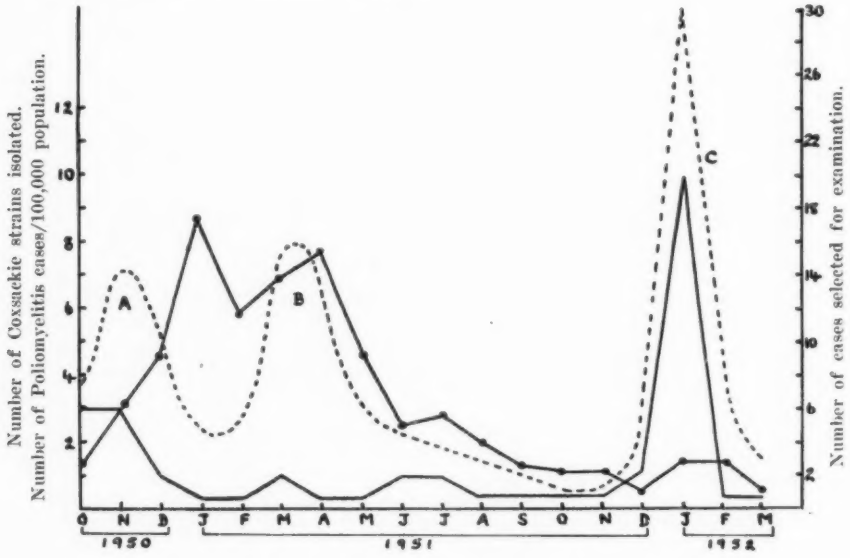
Number of Coxsackie strains isolated.
Number of Poliomyelitis cases/100,000 population.
Number of cases selected for examination.
A
B
C
O N D J F M A M J J A S O N D J F M
1950
1951
1952

Fig. 1. •—•—• Incidence of poliomyelitis in metropolitan area.
——— Number of Coxsackie strains.
- - - - - - Number of cases examined.

The nature of the infection in humans.

During the outbreak no fatal case of encephalitis was observed. Some patients made an uneventful recovery, but others showed sequelae lasting many weeks. It is not the purpose of this communication to cite the clinical histories of each patient. These will be published elsewhere. With the exception of cases K and L (diagnosed as ? poliomyelitis), each case was diagnosed as "encephalitis" by the clinician. This diagnosis was made without knowledge of, and, in most cases, many weeks before the findings of the laboratory investi-

TABLE 1.

	Hospitalized patients																					Sick contacts				
Patients	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	**V**	**W**	**X**	**Y**	**Z**	**P.c.**
Age	9	20	25	24	9	14	27	8	19	18	18	19	23	17	10	8	18	35	14	10	26	22	21	27	24	21	
Sex	M	F	F	M	M	M	F	M	F	F	F	F	F	F	M	M	M	M	M	M	F	F	F	M	F	F	
Virus recovered	—	+	—	+	+	+	+	—	+	+	—	—	+	+	—	—	+	—	+	—	—	0	0	0	0	0	52
Antibody present	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	+	100
Pyrexia	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Headache	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Stiff neck	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	95
Pleocytosis in C.S.F.	+	—	+	—	+	+	—	—	+	+	—	+	+	—	+	+	+	+	+	+	+	+	0	0	0	0	73
Muscle pain	+	—	+	—	—	—	+	+	+	—	+	+	+	—	—	—	+	+	—	—	+	+	—	—	—	+	55
Vomiting	+	+	+	—	+	+	—	+	+	—	—	—	—	—	+	+	—	—	+	—	+	—	+	—	—	+	50
Sore throat	—	—	+	+	+	+	—	+	+	+	—	+	—	—	—	—	—	+	—	—	—	—	—	+	+	—	41
Abdominal pain	—	—	—	—	—	—	—	+	—	—	—	+	+	+	+	—	+	—	—	+	—	+	+	—	—	—	36
Lassitude	+	—	—	—	—	—	—	—	—	+	—		—	—	+	—	—	—	+	+	+	+	—	+	+	—	36
Muscle weakness	—	—	+	+	—	+	—	—	+	—	—	+	—	—	+	—	—	—	—	—	—	—	—	—	—	—	27
No. of days in hospital	15	12	21	17	32	25	68	14	46	12	58	84	19	14	52	10	15	18	53	22	42	20	10*	12*	12*	14*	
Sequelae	+	—	—	—	+	+	+	—	+	+	—	+	+	+	+	—	—	—	+	+	+	+	—	—	—	+	63

0 = test not carried out. * = duration of illness.

gations became available. In Table 1 have been summarized the main clinical features associated with the outbreak. With one exception, the symptoms are given of only those patients whose clinical diagnosis was confirmed by virus isolation or by positive serological findings.

Not shown in the Table are several observations which merit attention. Almost 50 p.c. of the patients had been swimming and/or sunbathing a few days prior to onset. This estimate is probably on the low side, as the information on this aspect was not obtained from six patients. Sequelae were present in 63 p.c. of patients and the most common complaint was that of severe headache of many weeks' duration. Photophobia, nystagmus and dizziness were less common after-effects. Residual muscle weakness was apparent in four patients. The duration of hospitalization ranged from 10 days to 83 days with an average duration of 30 days. More females were infected than males. The average age of the males was 14 years and that of the females 21 years. The majority of females fell into the 18–22 years age group.

From subsequent examinations of contacts it became apparent that two or more individuals from at least seven families were infected with a group C Coxsackie virus. D and V were husband and wife whose only sick contact at time of onset was a female neighbour (age, 26 years). At the same time infants in each family developed sore throats. The infant of patient G also developed sore throat and pyrexia at the time the mother was admitted to hospital. The only symptomless contact giving a positive serological test for a group C Coxsackie strain was a male, aged 13½ years, the brother of patient T.

Failure to demonstrate other infective agents.

Rhesus monkeys were inoculated by the intracerebral route with ultracentrifuge deposits of the faeces of several patients (B, D, I, J, O). Nothing abnormal was detected in any of the monkeys inoculated. Adult mice inoculated by the intracerebral route with infected suckling-mouse brain from early passages showed no evidence of infection.

Properties of the infective agent.

The agent is filtrable, does not produce pocks on the chorio-allantois of 11-day old chick embryos and is pathogenic only for suckling mice. A comparison of the properties of Coxsackie groups A and B with the agent designated as group C is made in Table 2. The properties of the group C viruses are outlined below.

Histopathology. The only lesions found in suckling mice following intraperitoneal inoculation of infective material were in the central nervous system. The skeletal muscle appeared to be unaffected and pancreatitis could not be demonstrated. Lesions in the brain consisted of diffuse encephalitis, haemorrhages with perivascular cuffing, focal necrosis and pyknosis. The inflamma-

tory changes varied in intensity from animal to animal. These features differentiate group C viruses from groups A and B, which cause destruction of the skeletal muscle.

TABLE 2.

A comparison of the properties of Coxsackie viruses groups A and B with the newly-described group C.

		Coxsackie Group		
		A	B	C
Myositis		+	+	—
Encephalitis		—	+	+
Incubation period (days)		2–3	3–8	5–17
Serological type		numerous	few	one
Symptoms of infection in suckling mice		paralysis, spasticity, etc. (Die quickly.)	tremors, emaciation, wrist drop. (Die slowly.)	tremors, emaciation, wrist drop. (Die slowly.)
Association with disease in humans		herpangina, ? poliomyelitis	Bornholm disease	encephalitis
Resistance to germicides		markedly resistant to lysol, phenol, alcohol, ether, etc.	resistant to lysol, phenol, alcohol, ether, etc.	sensitive to lysol, phenol, 70 p.c. alcohol
Age susceptibility of mice (days)		up to 5 days	up to 5 days	up to 1 day
Concentration of virus in tissues	brain ID_{50}	10^{-6}	10^{-5}	10^{-2} to 10^{-4}
	muscle ID_{50}	10^{-7}	10^{-4}	$<10^{-1}$

Antigenic structure. The eight group C strains fall into one antigenic type. No relationship has been demonstrated with Coxsackie group A viruses or with group B types 1, 2 or a. The possibility of an antigenic overlap with group B type 3 is being investigated. The results of neutralization tests with hyperimmune mouse sera are given in Fig. 2.

Concentration of virus in tissues. The demonstration of virus in skeletal muscle of suckling mice infected with a group C strain after intraperitoneal inoculation is difficult. This finding is in marked contrast with the observations with group A and group B strains (see Table 2). The virus can be recovered from the brain more easily, but even then the ID_{50} is only between 10^{-2} and 10^{-3}, which is a lower concentration than we obtain for group A and group B strains.

Hyperimmune mouse serum against	Reciprocal of serum dilutions			
	20	80	320	1280
Group C	■	■	■	■
Group B 1	□	▨	▨	▨
Group B 2	□	▨	▨	▨
Group B 3	■	■	□	□
Group B 4	□	□	▨	▨

■ = 100 p.c. protection
□ = 100 p.c. deaths
▨ = test not done

Fig. 2.

virus strain	case no.	serum collected days after onset		reciprocal serum dilutions							
				1st specimen				2nd specimen			
		1st	2nd	20	80	320	1280	20	80	320	1280
K137	K137	6	111	■	■	◧	□	■	◧	□	□
W146	W146	6	36	■	■	■	■	■	■	■	■
L148	L148	15	46	■	■	■	□	■	■	■	□
A154	A154	11	19	■	■	■	◧	■	■	■	■
F155	F155	14	22	■	■	■	■	■	■	■	□
F155	S156	12	34	■	■	■	■	■	■	■	■
N162	N162	7	36	■	■	■	□	■	■	■	◧
A163	A163	6	44	■	■	◧	◧	■	■	■	■
E167	E167	31		■	■	■	□	▨	▨	▨	▨
K137	K150	8	34	■	◧	◧	□	■	■	■	◧
K137	B149	8	35	■	■	◧	□	■	■	■	◧

■ 100 p.c. protection
□ 100 p.c. deaths
▨ test not done

Fig. 3.

Fig. 2. Neutralization tests with a Group C virus (K137) against 4 serological types of Group B Coxsackie virus.

Fig. 3. Antibody response of patients to homologous virus or to another Group C strain, determined by suckling-mouse neutralization tests.

Age susceptibility of mice. This factor has proven to be of great practical importance in the carrying out of virus isolations and neutralization tests. It was early demonstrated that mice 48 hours old only exhibited symptoms of infection if very large doses of virus were given. Neutralization tests could not be performed unless every litter was 24 hours old or less. It soon became clear that some negative virus isolation results may have been recorded because the litters used on a Monday, for example, were 48 hours old. It is now our practice to discard litters older than one day. As an example, the group C strain K137 may be taken. This strain has now been passaged many times in suckling mice and has an ID_{50} for brain tissue which is higher than for many other group C strains. The results of inoculating the K137 strain by the intraperitoneal route into suckling mice of increasing age is shown in Table 3. Included for reference are a group A and a group B Coxsackie virus and a locally isolated herpes strain.

TABLE 3.

Virus	Age of mice in days: 1 M	1 D	2 M	2 D	3 M	3 D	5 M	5 D	21 M	21 D
Group A (W32)	8/8	3	8/8	3	8/8	3	8/8	3	0/8	S
Group B (E46)	8/8	3	8/8	3	8/8	3	8/8	4	0/8	S
Group C (K137)	5/6	6	1/7	13	1/6	10	0/7	S	0/8	S
Herpes (P29)	8/8	5	8/8	6	8/8	8	8/8	8	8/8	9

Column M { numerator = number of suckling mice paralysed or dying.
denominator = number of suckling mice in litter inoculated.
Column D = number of days to produce paralysis or death.
S = survived.

Incubation period. This varies with the concentration of virus and the route of inoculation. When approximately 100 ID_{50} of virus are given by the intraperitoneal route into mice no older than 24 hours, the first signs of infection may appear on the 5th or 6th days. With a slight reduction in the concentration of virus in the inoculum the incubation period may be increased up to seventeen days. The incubation period for group A strains is much shorter.

Symptoms of infection. There is a marked difference between suckling mice infected with group C strains and those infected with group A viruses. Mice infected with group A develop paralysis, many show spasticity and die promptly. Those infected with group C may first show emaciation followed by marked tremors, nearly always involving the tail and less often the head. Wrist drop is a predominant feature. Mice may show these features for many days before death occurs. The difference between group B and group C infected mice is not very great. The tremors and wrist drop appear to be more clearly marked and the delay in time of death longer with the group C strains. It is our experience that most suckling mice with histological evidence of involvement of the central nervous system have shown symptoms of tremors and wrist drop.

Resistance to germicides. Although the group C viruses are not as resistant to the effects of lysol and ethanol as the group A strains, strong suspensions are not completely inactivated after 1 hour's exposure to 70 p.c. ethanol or 1 p.c. lysol at room temperature. The virus is readily inactivated by 0·25 p.c. formalin.

Association with disease in humans. The strains of group C Coxsackie virus were closely associated with an explosive outbreak of a disease diagnosed as encephalitis occurring in mid-summer in Sydney at a time of low poliomyelitis incidence. Other groups of Coxsackie viruses have not yet been associated with an outbreak of this nature in Australia.

The antibody response of humans to infection with group C Coxsackie viruses.

The laboratory diagnosis of group C Coxsackie infections has been made by two methods, viz. (1) the isolation of the agent from the ultracentrifuge deposit of faeces during the acute stage of the disease followed by a demonstration of a rise in titre of antibodies to the agent isolated; or (2) the demonstration of a significant rise in antibodies to a group C virus during the course of illness. Fig. 3 shows the antibody response of patients to the homologous virus and, in cases where a strain was not recovered, to group C strain K137.

It can be seen that antibody appears soon after onset. It has been our experience with Coxsackie viruses that the serum collected from patients on the first day of admittance to hospital invariably contains antibody to the virus isolated from faeces or throat washings and that a rise in antibody cannot be demonstrated if serum is obtained much more than 12 days after onset.

The serum of ten patients diagnosed clinically as Bornholm disease contained no detectable antibody to Coxsackie group C strain, although showing significant rise in antibody titre to a Coxsackie group B strain (T80). Other sera from patients not showing typical symptoms showed no trace of antibody to the K137 strain when tested by the suckling-mouse neutralization test.

The presence of antibodies to group C viruses in pooled human serum.

Antibodies to Coxsackie viruses have been demonstrated in pooled human serum (P.H.S.) by Stanley *et al.* (1953b). The response to the outbreak caused by the group C Coxsackie viruses was illustrated in this paper. Antibodies were estimated by the suckling-mouse neutralization test which is more sensitive than the complement-fixation test (Stanley and Dorman, 1953). It was seen that antibodies could be detected a few weeks prior to the time when the outbreak was clinically recognized, and that a good response appeared in mid-January, coincidental with the highest incidence.

Antibodies are still present in pooled human serum collected during June, 1952.[1]

DISCUSSION.

The laboratory examinations of the outbreak described were carried out many weeks after its decline. As a result there was some delay and difficulty in obtaining suitable specimens from contacts It is not proposed to discuss the clinical diagnosis, which was made without knowledge of the investigations of the virus diagnostic laboratory. The findings are at least indicative of a virus infection of the central nervous system. It has not been proven that the Cox-

[1] Samples of pooled human serum were obtained through the courtesy of Dr. R. J. Walsh, Director, The Red Cross Blood Transfusion Service, Sydney.

sackie viruses isolated were responsible for the outbreak as none was isolated from central nervous system material. The circumstantial evidence (the isolation of one antigenic type of virus from the faeces in a large number of cases and the demonstration of antibodies to the virus in the patient's serum in all cases) is such as to indicate a very close relationship. It is realized, however, that the technique of monkey inoculation for the recovery of poliomyelitis viruses is by no means perfect and that some LEON strains may be missed.

As this report includes (with the exception of contacts) only hospitalized patients, an incomplete picture of the outbreak is presented. It is, however, believed that the outbreak was widespread in the Sydney metropolitan area, sufficiently so to cause comments in the lay press and a sudden rise in antibody in pooled human serum to the virus thought to be responsible for the outbreak. The demonstration of antibodies to the etiological agent in pooled human serum immediately prior to the time when the disease first became clinically apparent may be explained by assuming that there was an increase in the number of carriers of the infective agent at this time. If a similar process operates with other virus diseases, and if a sensitive enough test for antibodies is available, then it may be possible to predict an increase in the number of subjects carrying the agent, irrespective of whether that number reaches epidemic proportions or not.

The infective agent was shown to be present only in the faeces during the course of the disease and could be recovered as long as 20 days after onset. Its presence was not demonstrated in throat washings. Two specimens of C.S.F. were inoculated intracerebrally into adult mice at the time of the outbreak. These and other specimens of C.S.F. were not saved, and, as a result, no attempt was made to recover the virus from that source. As there were no fatal cases other material from the central nervous system was not available.

The agent was filtrable and was shown to share some of the properties of the Coxsackie group of viruses. Because all strains tested produced infection *only* in suckling mice with resulting lesions in the central nervous system, it was assumed that the agent was a Coxsackie virus. Further investigation of the properties revealed that all strains isolated differed slightly from the group B Coxsackie viruses and differed markedly from the group A Coxsackie viruses so far isolated. These differences associated with the newly described infective agent are:

(1) The lower concentration of virus in the C.N.S.
(2) The insusceptibility to infection of suckling mice older than 24 hours.
(3) The greater susceptibility of the viruses to germicides.
(4) The absence of virus and lesions in skeletal muscle.

It was considered that these differences were sufficient to warrant a temporary designation of Coxsackie group C to the viruses. This, however, may

be criticized on the grounds that an antigenic overlap has been demonstrated with group B, type 3[2]. The alternative would be to designate the agents as Coxsackie group B type 5 or 6, depending on the number of existing antigenic types of group B. The term "group C" is preferred as a temporary measure until the matter can be clarified.

The antibody response of patients, as demonstrated by suckling-mouse neutralization tests, indicates that antibody appears early in the disease and that a rise is difficult to demonstrate unless serum is collected within one week of onset. Antibody to titres between 1/80 and 1/320 could be demonstrated in sera four to five months after onset.

One interesting feature of the outbreak, as illustrated in Fig. 1, was the time of its occurrence in relation to the incidence of poliomyelitis. It is possible that the encephalitis type of Coxsackie virus exerts a retarding or interfering action on poliomyelitis (Dalldorf, 1951; Stanley, 1952), and it has been observed (Dalldorf, personal communication) that infections (e.g. Bornholm disease) due to this type of Coxsackie virus occur at a time when the morbidity and mortality rates of poliomyelitis are low.

Monkeys were inoculated by the intracerebral route with several specimens of faeces (ultracentrifuge deposits). No evidence for the presence of a poliomyelitis virus was obtained by this method.

SUMMARY.

An account is given of the properties of a virus, associated with an explosive outbreak of mild encephalitis in the Sydney metropolitan area in midsummer. There were no fatal cases. The virus was shown to be a Coxsackie virus which possessed properties sufficiently different from the known groups, A and B, to warrant a temporary designation of Coxsackie Group C.

Acknowledgment. The authors are grateful to the Medical Superintendent of the Royal Alexandra Hospital for Children for making available the faeces, serum and clinical notes of four of the patients referred to in this paper.

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[2] Serum provided through the courtesy of Dr. Gilbert Dalldorf, Director, Laboratories and Research, New York State Department of Health, Albany, N.Y., U.S.A.

STUDIES ON INTERMEDIARY IRON METABOLISM

I. MODIFICATIONS OF METHODS FOR MEASURING SERUM IRON AND IRON BINDING CAPACITY OF SERUM

by I. KALDOR[1]

(From the N.S.W. Red Cross Blood Transfusion Service, Sydney).

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The experiments of McCance and Widdowson (1937) and Hahn *et al.* (1939) have shown beyond doubt that the mammalian organism is unable to excrete significant amounts of iron. The total daily loss is 1 mg. or less in man (Drabkin, 1951). It is known on the other hand, that 25 mg. of iron are liberated daily within the body from catabolized haemoglobin only. The same quantity of iron must be used over the same period for the synthesis of new haemoglobin molecules. The reutilization of iron is based on a mechanism of storage and transport.

The iron engaged in haemoglobin metabolism is some 75 p.c. of the total body iron. 15–20 p.c. is in the storage fraction and the remaining 5–10 p.c. in myoglobin and the haem-containing enzymes. The last mentioned fraction plays a relatively unimportant part in iron metabolism. Storage iron is mainly in the form of ferritin, a colloidal iron-protein compound, located in the liver, the spleen and the kidney (Granick, 1946). Normally haemoglobin iron is in dynamic equilibrium with the storage fraction. Iron is mobilized from the storage organs when required and is deposited in them again when the need for the additional active iron has ceased. When the equilibrium is undisturbed, the same number of iron molecules is deposited in the storage fraction over a given period of time as the number of molecules dispatched from it.

Serum iron had been regarded for some time as the transport iron fraction. The nature of the transport mechanism was put in a new light by the work of Schade and Caroline (1946) and Laurell (1947). They isolated and characterized a specific serum protein which serves as the carrier of serum iron, just as haemoglobin is the carrier of oxygen (Laurell, 1951). Iron forms a complex with this protein (called transferrin by Laurell) in preference to any other serum protein present. The reaction is reversible. The normal iron binding capacity of 100 ml. of serum is 300 μg. of iron and the average serum iron concentration is only 100 μg. of iron per 100 ml. of serum. Consequently if required the transport system can carry three times the amount of iron it normally carries to meet physiological requirements.

[1] This work was undertaken during tenure of a Fellowship of the National Health and Medical Research Council.

It has been shown (Laurell, 1947; Finch *et al.*, 1949) that certain physiological and pathological variations of serum iron and of iron binding capacity values can be correlated with events of the iron storage and iron utilization mechanism.

This paper describes methods for determining serum iron and iron binding capacity of serum.

Materials and Methods.

A. Serum Iron.

Principle of the method of determination.

Dissociation of the iron-transferrin complex is brought about by acidifying serum with hydrochloric acid. The serum proteins are then precipitated with trichloracetic acid and a clear supernatant obtained by centrifuging. The pH of the supernatant is adjusted to pH 4·6, the iron reduced to Fe^{++} with thioglycollic acid and finally *ortho*-phenanthroline added. The red colour of the Fe^{++} -*ortho*-phenanthroline complex is measured colorimetrically. The procedure is a modification of the original method of Kitzes *et al.* (1944).

Reagents.

8 p.c. hydrochloric acid

40 p.c. trichloracetic acid, redistilled

1 p.c. *para*-nitrophenol

Concentrated ammonium hydroxide solution and a series of dilutions ranging from 2: 5 to 1: 100.

Acetate buffer, pH 4·58, 27·2 ml. glacial acetic acid +33·4 gm. anhydrous sodium acetate reagent dissolved in 250 ml. distilled water.

Thioglycollic acid, B.D.H. reagent.

2 p.c. *ortho*-phenanthroline in pH 4·58 acetate buffer solution.

A stock solution of iron was prepared from iron wire reagent and its concentration checked by gravimetric analysis. Standard solutions for calibration purposes are diluted from the stock solution from time to time, as required.

All reagents must be of analytical purity. Solutions are kept in Pyrex glass containers. Distilled water is produced in an all glass still. All glassware is washed in a hot detergent solution, rinsed with hot water, immersed in a 2·5 p.c. hydrochloric acid bath for 24 hours and finally rinsed with double distilled water.

Apparatus.

A photoelectric colorimeter used in this work was constructed in the workshop of the New South Wales Red Cross Blood Transfusion Service. A 2·2 volt lens front torch light bulb is used as the source of transmitted light. The current is drawn from a 6-volt battery and two potentiometers are included in the circuit for adjustment of light intensity. The bulb, the potentiometers and an Eel photocell are enclosed in a metal box. A solid block fitted between the bulb and the photocell is shaped to accommodate cuvettes and filters. The photocell is connected through a switch to a Cambridge spot galvanometer of 450 ohm internal resistance. The colour of the Fe^{++} —*ortho*-phenanthroline solution is measured in matched Ogal cuvettes (length of light path 135 mm.) with an Eel No. 623 lightfilter (maximum transmission at 490 mμ).

Preliminary experiments.

In the original method of Kitzes *et al.* (1944) 3–5 ml. of serum are used for the analysis. The serum proteins are denatured by boiling. No hydrochloric acid is used. 2 ml. of 25 p.c. trichloracetic are added, the mixture boiled, stirred, centrifuged and the supernatant decanted. Boiling, stirring and centrifuging are repeated after adding 4 ml. distilled water and 1 ml. trichloracetic acid to the precipitate. The resulting supernatant is united with the supernatant of the first extraction.

This procedure has several drawbacks. The repeated extraction is time consuming, and is an additional source of error. Filtration is necessary to obtain a clear supernatant. Filter paper used for this purpose must be specially treated to render it iron free.

The reproducibility of the results obtained with the original technique was poor. Undoubtedly, results were improving with practice and more rigid standardization of the technique, yet the disadvantages mentioned were overcome only after having made certain modifications. Rapid dissociation of the iron-transferrin complex in native serum can be obtained by lowering the pH (Laurell, 1947). It was found that if hydrochloric acid is added to serum and the proteins subsequently precipitated by trichloracetic acid, all the iron appears in the supernatant which is a clear fluid after centrifuging and can be removed without filtration. The original method of Kitzes *et al.* was retained for the subsequent steps of preparing the supernatant for colorimetry.

TABLE 1.

Procedure of extraction and preparation for colorimetry.

(1) Serum	(2) Distilled water	(3) 8 p.c. hydrochloric acid	(4) 40 p.c. trichloracetic acid	(5) Distilled water
2·5 ml.	0·5 ml.	0·5 ml. Shake vigorously. Leave at room temperature for 30 minutes. Place in 90° C. waterbath for 5 minutes. Cool	2·0 ml. Stir. Place in 90° C. waterbath for 10 minutes. Stir. Replace in waterbath for 10 minutes. Cool.	To make up to 6 ml. Centrifuge for 20 minutes at 2,000 r.p.m.

An aliquot of the clear supernatant obtained at (5) is taken for colorimetry.

(6) Aliquot*	(7) (a) 1 p.c. *para*-nitrophenol	(7) (b) Ammonium hydroxide	(7) (c) pH 4·58 acetate buffer	(8) Thioglycollic acid	(9) 2 p.c. ortho-phenanthroline	(10) Distilled water
4·5 ml.	2 drops	Add dropwise until solution turns pale yellow	0·5 ml.	2 drops	0·1 ml.	To make up to 6 ml. Mix thoroughly by inverting tube three times.

* Replaced with different volumes of a standard iron solution for calibration purposes.

The modified procedure.

2·5 ml. samples of serum are taken for analysis. The procedure is carried out in ½-in. diameter Pyrex test tubes graduated at 0·5 ml. intervals. These tubes are calibrated to conform to B class standard and the graduation marks are full rings. Details of the procedure are shown in Table 1. The successive steps of the procedure are numbered 1–10.

The test tubes are kept stoppered throughout and opened only when necessary. Glass stirring rods are used for stirring at (4) and for mixing at (7).

The volumes shown at (1), (2), (3), (6) as well as (5) and (10) are pipetted with Pasteur pipettes, the quantities being measured by the graduations on the tube. Colorimetry is performed 20 minutes after completing the procedure.

The colorimeter is calibrated with standard iron solutions of varying concentration prepared in the graduated test tubes as described in Table 1 (6) to (10). The final concentration of iron in the tubes is 0·125–1·125 μg. Fe per 1 ml. of solution. A reagent blank solution is prepared with distilled water. The solutions are read in the colorimeter against distilled water. The transmission readings are converted into density values using the formula: D = 2 — log p.c. transmission. The density value of the reagent blank is subtracted from the density value of each standard solution to correct for iron impurities present in the reagents. The equation of the linear relationship between concentrations and densities is calculated. A conversion constant (K) is obtained from this equation.

Four solutions are prepared for the colorimetry of serum iron. The first (S) is prepared with all the steps shown in Table 1. The second (SB) is similarly prepared but distilled water is substituted for *ortho*-phenanthroline. In the preparation of the third (R) the serum is replaced by distilled water. The fourth (RB) is identical with the third (R) except that distilled water displaces ortho-phenanthroline as well as serum. Each solution is read in the colorimeter against distilled water. The density value representing serum iron D_{SI} is derived from

$$D_{SI} = (D_S - D_R) - (D_{SB} - D_{RB})$$

where $(D_S - D_R)$ is the correction for iron impurities in the reagents and $(D_{SB} - D_{RB})$ gives a correction for non-specific absorption apparently related to the faint yellow pigmentation of the serum extracts.

This system of blank solutions gives slightly more accurate correction and requires less manipulation than the original one.

The serum iron value is calculated as μg. of iron per 100 ml. of serum from the corrected density value (D_{SI}) the conversion factor (K) and the dilution factor of the procedure (F).

$$\text{SI } \mu\text{g./100 ml.} = D_{SI} \times K \times F$$

For the given set of conditions the value of K is 4·2553, F = 320.

Experiments with the modified method.

(a) A series of measurements was performed to determine if appreciable amounts of iron were left in the serum precipitates after a single extraction with trichloracetic acid. The once extracted precipitates of 8 sera were extracted a second time with 2·0 ml. of trichloracetic acid. The supernatants resulting from the two extractions were prepared separately for colorimetry. Results are shown in Table 2.

The overall increase after the second extraction is 2 p.c. It should be noted, however, that Nos. 7 and 8 are outside the range of normal serum iron values and if these two were omitted the calculated overall increase would be even smaller.

(b) The error of the method was estimated in a series of duplicate determinations combined with recovery tests. The serum iron values of 11 sera were measured in duplicate. The value was also measured on an iron-enriched portion of each serum. The difference between the duplicate values was expressed as the percentage deviation from their mean.

TABLE 2.

Fractional extraction of serum iron.

Serum	μg. Fe/100 ml. serum	
	Measured in the first extract	Measured in the second extract
1.	87	2
2.	98	—3
3.	109	0
4.	112	—3
5.	212	5
6.	214	5
7.	248	8
8.	253	10
Total	1333	24
Mean	166·7	3

TABLE 3.

The error of the serum iron determination.

	Reproducibility of duplicates			Recovery tests			
	Serum iron μg. Fe/100 ml. serum			Iron added	Total iron in the enriched serum		
Serum	Duplicates	Means	Deviation p.c.	μg. of Fe	Calculated	Measured per 100 ml. of serum	Difference p.c.
1.	46 46	46	0	60	106	107	+1
2.	46 52	49	±6·6	60	109	108	—1
3.	51 52	52	±0·95	60	112	103	—8
4.	52 53	53	±0·95	60	113	116	+3
5.	69 69	69	0	60	129	128	—1
6.	77 81	79	±0·5	60	139	140	—1
7.	89 95	92	±3·3	60	152	158	+4
8.	99 105	102	±3·0	60	162	159	—2
9.	110 110	110	0	60	170	176	+3
10.	120 125	123	±2·0	60	183	188	+3
11.	159 164	162	±1·5	60	222	225	+3

The difference between the calculated and the measured concentration of iron of enriched sera was expressed as the plus or minus percentage difference from the calculated value. Results are given in Table 3. These results show an average error of about 2 p.c.

(c) The ratio of serum iron to haemoglobin iron is approximately 1 : 1000 in normal whole blood. The breakdown of relatively small amounts of haemoglobin in the course of extracting the iron from the serum would therefore lead to extensive errors. Experiments on haemolysed serum have shown that haemoglobin precipitates with the serum proteins. (The precipitate stains pink). Direct evidence indicating that no haemoglobin iron is liberated during the procedure was obtained on three samples of blood. The blood was allowed to clot and the tube then centrifuged. A clear serum aliquot was carefully removed from the clot and the clot then stirred. After further centrifuging another serum aliquot containing free haemoglobin was removed and serum iron values determined on both. There was no difference between the serum iron values of the aliquots of any of the three samples.

B. Serum Iron Binding Capacity.

The serum iron binding protein, transferrin, has been shown to bind iron in the ratio of 2 molecules of iron to 1 molecule of transferrin. The formation of the iron-transferrin complex is accompanied by the development of a reddish colour both in a solution containing the purified transferrin and in native serum (Holmberg and Laurell, 1945, Schade and Caroline, 1946, Laurell and Ingelmann, 1947, Surgenor *et al.*, 1949). The colour change can be measured and is proportional to the number of iron-transferrin complex molecules formed. This is the basis of the method developed by Rath and Finch (1949) for measuring the iron binding capacity of serum.

It must be repeated at this point that what is referred to as the iron binding capacity of serum is actually the iron binding capacity of the transferrin present. The iron is firmly bound in the complex and reacts with iron reagents only at low pH, when the complex dissociates. On the other hand iron added to the serum in excess of the iron binding capacity reacts readily with *ortho*-phenanthroline at neutrality. This latter reaction was used by Laurell (1947) for measuring the iron binding capacity.

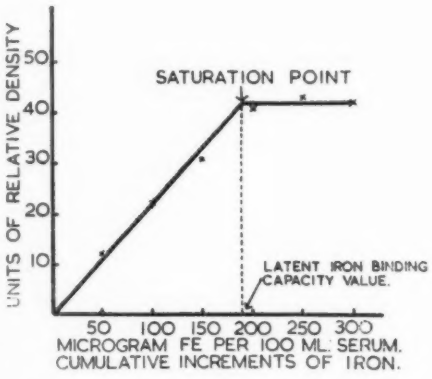

Fig. 1. The determination of the latent iron binding capacity value.

The method of Rath and Finch was used in the present work. One colorimeter cuvette is filled with 5 ml. of 0·9 p.c. saline, and another with a mixture of 3 ml. of saline and 2 ml. of the serum to be tested. To both cuvettes 0·05 ml. quantities of a standard iron solution are added repeatedly, each addition representing an increment of of 50 μg. of iron per 100 ml. of serum. The contents of the cuvette are stirred with a glass rod after each addition of iron solution. Readings are made in the colorimeter 2–3 minutes after each mixing, with the saline solution as blank. A light filter with a transmission peak at 520 mμ is recommended. The iron solution is added until three successive readings show no further change in the colorimeter reading. Two straight lines were fitted to the plotted

points as shown in Fig. 1. Projection of the intersection on the abscissa gives the numerical value of the latent iron binding capacity. The total iron binding capacity of serum is the sum of the measured serum iron and the latent iron binding capacity. This relationship is illustrated in Fig. 2.

The validity of this method has been proved by Rath and Finch in experiments with purified transferrin. Values obtained by the present author on duplicate serum samples agree to within 30 μg. in the majority of sera examined. In some 30 p.c. of the sera examined the density failed to assume a constant value on reaching the saturation point. This anomaly made determination of the saturation point uncertain (see Fig. 3, curve 1) but could be overcome by using serum fully saturated with iron as the blank solution.

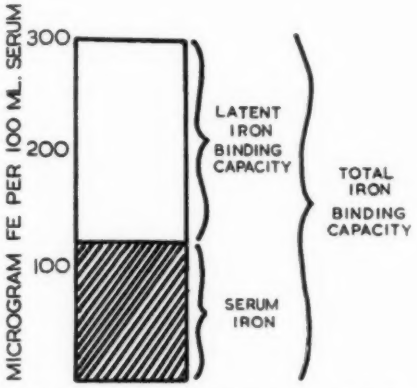

Fig. 2. The subdivisions of iron binding capacity.

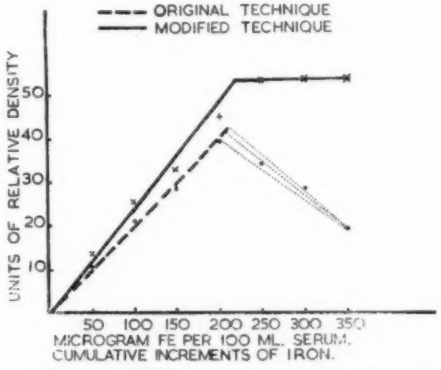

Fig. 3. Determination of the iron binding capacity on a serum sample unsuitable for the original technique.

This modification is carried out in the following manner. Each cuvette is filled with 2 ml. of serum and 2 ml. of saline. 400 μg. of iron dissolved in 0·02 ml. of distilled water is added to one cuvette (the blank) and the same volume of distilled water to the cuvette holding the test solution. The transferrin of the blank solution has now been saturated with iron and its density is higher than that of the test solution. The blank cuvette is placed in the colorimeter and the intensity of transmitted light adjusted to give a galvanometer reading of 80. Using this arbitrary setting for the blank the test solution should read above 80. Standard iron solution is added to both cuvettes as in the original method. The saturation point is reached when three successive galvanometer readings are unchanged and identical for both test and blank solutions. Curve 2 in Fig. 3 was obtained with the modified technique on the same serum which gave curve 1 with the original technique.

Duplicate determinations of the iron binding capacity were performed on a number of sera with both techniques and values were found to agree within the limits of experimental error.

SUMMARY.

A modification of the method of Kitzes *et al.* for measuring serum iron is described. Modifications concern the preparation of the protein free iron extract and the system of blank solutions used in the colorimetry of iron with *ortho*-phenanthroline. The reproducibility of duplicate measurements and the error of the recovery of iron are of the order of 2 p.c. over the range of physiological serum iron values.

A modification of the method of Rath and Finch for the determination of the iron binding capacity of serum is described. A sharp end point in the determination could be obtained on more sera with the modification than with the original method.

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STUDIES ON INTERMEDIARY IRON METABOLISM

II. VARIATIONS OF THE SERUM IRON VALUE IN BLOOD DONORS AND CONTROL SUBJECTS

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(Accepted for publication 6th October, 1952.)

Recent investigations (Laurell, 1947; Finch *et al.*, 1950) have demonstrated the importance of serum iron—the protein bound non-haemoglobin iron of the blood—in intermediary iron metabolism. Serum iron values in healthy subjects have been studied by a number of workers and others have investigated the physiological variations (Moore *et al.*, 1939; Vahlquist, 1941; Waldenstrom, 1946; Laurell, 1947; Totterman, 1949).

A well-established difference exists between the sexes, similar in direction to and of the same order as the sex difference in haemoglobin and in red blood corpuscle concentration. Variation occurring in the individual from day to day was reported to be the same as the variation between individuals, i.e. ±30 p.c. of the mean value (Totterman, 1949). It is difficult to form a clear picture from the available data on the subject of diurnal variation. Recently Hamilton *et al.* (1950) have found a regular diurnal variation characterized by high morning, low evening values in 12 individuals. This is in conformity with the trend observed by other workers (Vahlquist, 1941), but Moore *et al.* (1949) found irregular fluctuations and Wetzel was quoted by Totterman (1949) to have found a reverse trend.

This paper describes the results of serum iron measurements made on blood donors. The effects of repeated blood donations were investigated in individuals of different age groups and of both sexes and the diurnal variations were also assessed.

Materials and Methods.

The blood donors examined were members of the voluntary donor panel of the N.S.W. Red Cross Blood Transfusion Service and represent a cross section of the healthy population. They are asked to donate blood once every three months and are questioned regarding their state of health since their previous visit. Any donor suffering from an infection, including coryza is rejected. The haemoglobin concentration of each donor's blood is measured before blood is removed. The minimum requirements for males and females are 13·0 gm. and 11·8 gm. Hb per 100 ml. blood respectively.

Subjects referred to in this paper as "donors" have given 400–450 ml. of blood on each of ten or more occasions and some have acted as donors up to 43 times. The average number

[1] This work was undertaken during tenure of a Fellowship of the National Health and Medical Research Council.

of donations varied between 19 and 23 for the different donor groups shown in the Tables. The control subjects or "controls" were recently enrolled donors who had given blood only once or twice previously.

Samples for assay were collected through the needle and rubber tubing which had been attached to the transfusion bottle. All samples were collected after 400–450 ml. of blood had passed through the tubing and were run into test tubes previously treated to remove traces of iron. The blood was allowed to clot and the serum separated by centrifuging. Serum iron assays were performed by a modification (Kaldor, 1953) of the method described by Kitzes *et al.* (1944).

EXPERIMENTAL RESULTS.

Table 1 shows the mean serum iron values of 73 donors and 75 controls grouped according to sex. Samples were collected at various times between 9 a.m. and 4 p.m. The values shown are not comparable with serum iron values quoted as normal by other workers because the latter always refer to blood collected in the morning from fasting subjects.

TABLE 1.

Serum iron values of donors and controls measured on samples collected between 9 a.m. and 5 p.m.

Group	Control (75 subjects) μg. Fe. per 100 ml. serum (Mean ± Standard Error)	Donors (73 subjects)
Males (65 subjects)	97±6	95±5
Females (83 subjects)	82±5	73±6

The difference between the mean serum iron concentration of donors and of controls is not statistically significant in either sex, although the difference between the two female groups indicates a certain trend which will be discussed later.

The same data are further analysed in Table 2. Here the values are arranged in three groups according to the time of day at which blood was taken.

TABLE 2.

Serum iron values (μg. Fe. per 100 ml. serum) grouped according to time of collection of samples.

Time of collection		Males		Females	
		Controls	Donors	Controls	Donors
9 a.m. to 11 a.m.	Number of subjects	10	6	14	10
	Mean ± standard error	*120 ± 10*	*130 ± 17*	*96 ± 13*	*77 ± 8*
	Standard deviation	33	42	49	26
	Average age	30 years	30 years	32 years	46 years
11 a.m. to 1 p.m.	Number of subjects	11	10	13	13
	Mean ± standard error	*90 ± 6*	*93 ± 6*	*81 ± 11*	*73 ± 10*
	Standard deviation	20	18	39	34
	Average age	32 years	40 years	30 years	44 years
1 p.m. to 4 p.m.	Number of subjects	14	14	13	20
	Mean ± standard error	*85 ± 7*	*81 ± 7*	*67 ± 6*	*71 ± 7*
	Standard deviation	35	38	21	31
	Average age	30 years	38 years	32 years	41 years

The figures show the gradual decline of the serum iron value during the day. The difference between morning and afternoon values is statistically significant in the male control group and statistically probable in the male donor and female control group. The differences between donors and controls are not statistically significant in either sex. However, the morning value of female donors is markedly lower than the corresponding value of the control group and as a result the diurnal decline is not apparent in the female donors. The average age of the female donors was higher than that of the controls and it was thought that this might account for the differences.

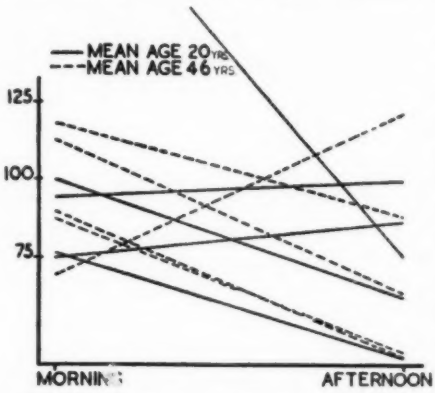

Fig. 1. Serum iron values of 11 female subjects measured on samples collected in the morning and in the afternoon.

To test the consistency of the diurnal variation and also the effect of age on this variation, serum iron values were determined at 9 a.m. and again at 5 p.m. on the same day on 11 female subjects. The results are shown in Fig. 1. Only one of the six females in the higher age group had a higher serum iron value in the afternoon than in the morning but two of the five young females showed a moderate rise in the afternoon. These results show that a consistent trend is not observed in all subjects and the mean values for female subjects in Table 2 may be due to chance selection.

These experiments also showed that the serum iron value varies within wide limits as is indeed reflected by the high value of the standard deviations in Table 2. Actually seven subjects, including one male, showed serum iron values below 40 μg.

DISCUSSION.

Blood donors. Analysis of the material fails to show any significant difference between the serum iron values of regular blood donors and those of healthy control subjects. This is contrary to Laurell's findings (1947). The mean serum iron value of 17 donors examined by him was lower than the mean value of a group of control subjects. Close study of his data reveals that the donors had given an average of 2,500 ml. of blood during the twelve months prior to Laurell's investigation. This is 50 p.c. more than the maximum amount of blood taken from our donors over a similar period. The time which had elapsed between the last donation of Laurell's donors and the performance of the serum iron assay was shorter (2 months) than the corresponding time in our

material (3 months). These considerations may explain the low serum iron values found in Laurell's donors.

The average number of donations in our material was 20, which corresponds to the removal of a total of 8,000 ml. blood evenly spread over a period of five years. Several of the subjects had donated twice this quantity. The amount of iron lost in the haemoglobin of 400 ml. of blood is some 200 mg. This quantity has to be replaced by absorption in 90 days (the interval between two donations), to maintain the physiological concentration of iron in the body. The average amount of iron absorbed normally is 0·9 mg. per day (Drabkin, 1951), i.e. a more than twofold increase in iron absorption must be maintained by these donors over an indefinitely long period. The absence of any significant change in the level of serum iron suggests that iron metabolism is readily adapted to increased demands.

Sex difference. On pooling the values of blood donors and controls, mean morning figures of 124 and 88 μg. are obtained for the two sexes. These still cannot be regarded as the highest daily values. Experimental material not included in this paper yielded mean values of 135 and 110 μg. for the two sexes on blood collected from subjects between 8 and 9 o'clock in the morning. A sex difference of about 20 p.c. is well maintained during the day.

Diurnal variations. A definite diurnal variation of the same magnitude as that found by Hamilton *et al.* (1950) is seen in all groups with the exception of the female donor group.

The inconsistent trend in individuals shown in Fig. 1 raises the question as to whether the absence of diurnal variation in the female donor group (Table 2) might not be due to chance selection of subjects. There is, however, no available information to indicate the percentage of individuals who show the reverse diurnal trend. On the other hand, the female donor group, with the higher mean age, may be subject to endocrinal disturbances associated with the menopause. Chaloupka *et al.* (1951) measured the serum iron concentration of 275 women, who were between the ages of 17 and 86 years and found a significant decline of the value with advancing age. They also found a slightly lower mean value during the menopause than immediately before or after. Furthermore, Hamilton *et al.* (1950) were able to show in experiments on rats and dogs that the serum iron level is related to adrenocortical activity. In the light of these findings the low morning value of female donors and the resulting virtual absence of diurnal variation in this group could possibly be attributed to endocrine disturbances in the particular age group due to the menopause. That this is not the complete explanation is shown in the inconsistency of the diurnal variation found in those subjects who were tested both in the morning and in the evening of the same day (see Fig. 1).

Low serum iron values. The seven subjects who gave serum iron values below the recognized physiological range were all considered to be normal sub-

jects. Their haemoglobin values were not reduced. Five of the subjects, including one male, were tested in the afternoon and the values recorded may represent an exaggeration of the diurnal decline. The practical importance of these findings concerning the diurnal variation is that a single determination of the serum iron which gives a value of less than 40 μg. should not be regarded as an indication of abnormality of iron metabolism.

SUMMARY.

The serum iron concentration was measured in blood donors and in control subjects. No significant difference was found between the values of the two groups. Higher values were found in the morning than in the afternoon in both sexes, but this tendency was less apparent in the group of female blood donors. There was a considerable difference between the average age of this group and that of its control group (43 years and 31 years) and this may explain the anomaly. The mean values of the female groups were about 20 p.c. lower than those of the male groups, both in the morning and in the afternoon.

Acknowledgment. The author is indebted to Miss Nancy Davies for continued and efficient assistance with the technical aspects of this work.

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THE EFFECT OF FOREIGN PLASMA ON CAPILLARY PERMEABILITY IN UNSENSITIZED ANIMALS

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While investigating the absorption of plasma proteins from the peritoneal cavity of rats Courtice and Steinbeck (1951) observed that fresh foreign plasma caused an exudation of fluid containing protein into the cavity whereas homologous plasma did not. They concluded that the foreign plasma increased capillary permeability. This effect obscured the considerable lymphatic absorption of protein, which occurred concurrently with the exudation and could be demonstrated by labelling the injected protein with the blue dye, T1824. It had led to an underestimation of the rate of absorption of protein-rich fluid from the peritoneal cavity by many previous workers who had injected foreign plasma and measured the volume of fluid remaining after various intervals. In fact, as Courtice and Steinbeck showed, homologous plasma was rapidly absorbed after intraperitoneal injection, slightly more rapidly, indeed, than 0·9 p.c. sodium chloride solution.

The aim of the experiments reported in this paper was to determine if the effect of foreign plasma on capillary permeability was a general one, by increasing the number of species employed as donors of the foreign plasma and as recipients of the injection, and by injecting plasma into the pleural cavity and skin as well as into the peritoneal cavity. Previous experiments had suggested that the damage to capillaries caused by foreign plasma lasted only a few hours, although large amounts of exudate might be produced in that time. Further data on this point are now presented.

METHODS.

Experiments on serous cavities. Twenty ml. heparinized plasma/kg. body weight was the amount used for intraperitoneal injections, with 6 ml./kg. for intrapleural injections. The method of injection and the general plan of experiments have been previously reported (Courtice and Steinbeck, 1950 and 1951; Courtice and Simmonds, 1949). In most cases the blue dye, T1824, from the same stock bottle used in the previous work, was added to the plasma before injection, thus labelling the plasma proteins. The dose of dye was the same as in previous experiments, viz. 4 mg./kg. body weight.

[1] This work was carried out with the aid of a grant from the National Health and Medical Research Council, Canberra.

Intradermal tests. The tests were performed in anaesthetized rats and rabbits, using the abdominal skin. The hair was clipped short with scissors and smoothed down with liquid paraffin. After intravenous injection of T1824, 6–8 mg./kg. body weight, to label the circulating plasma proteins, 0·1–0·2 ml. of the substances to be tested was injected under the epidermis with a fine gauge hypodermic needle. Duplicate or triplicate injections of test substances were always given in each animal and its own serum or heparinized plasma injected as a control. These were read after ½, 1½ and in some cases 3 hours.

Analytical procedures have been previously described (Courtice and Steinbeck, 1950).

RESULTS.

Intraperitoneal injections of foreign plasma.

In rats. Table 1 shows that considerably more fluid is recovered from the peritoneal cavity at various intervals after injection of sheep, dog, and horse plasma than after homologous (rat) plasma. Thus these foreign plasmas, like those of human, bovine, and rabbit species are seemingly absorbed more slowly than homologous plasma. With all except the human variety, substantial amounts of fluid were recovered from the pleural cavities. With all the foreign plasmas tested there is, therefore, evidence of capillary damage with exudation. Comparison of the average results (Fig. 1, A and B) suggests that plasmas from different species varied in regard to the amount of peritoneal exudate induced and the volume of fluid which leaked out of lymphatics into the pleural cavities as it passed up from the diaphragm (see Courtice and Steinbeck, 1951). Too few samples of most species have been tested to eliminate variation in effect of individual plasma. In the case of human and rabbit

TABLE 1.

Volumes of fluid recovered, in ml./kg., from the peritoneal and pleural cavities after intraperitoneal injection of foreign plasma, 20 ml./kg., in rats. Averages for groups of 4 to 5 rats.

Type of plasma injected	Volume of fluid recovered		Hours after injection
	Peritoneal	Pleural	
Sheep 1	10·7	5·3	5
Sheep 2	12·6	4·2	3
	7·8	14·1	7
Dog 1	3·0	6·2	5
Dog 2	20·1	1·7	3
	6·9	5·0	5
Horse 1	12·1	0·3	3
	2·3	1·4	5
Horse 2	13·8	0·9	3
	7·3	1·6	5
Rat	1·4	0·2	3
	0·1	0·3	5

plasma where volumes have been measured at 3 hours and 5 hours after injection of samples from 3 to 10 different donors or pools there is a marked and statistically significant species difference (t corresponds to $p<0{\cdot}01$ at 3 hours and 5 hours). The curves suggest a gradation of effects, from the foreign plasmas which had a marked effect, such as bovine and rabbit, to those which had but a moderate effect, such as horse and guinea-pig. Human plasma produced intense capillary damage, as shown by the gross exudation of fluid and protein and the petechial haemorrhages seen under the serosa at autopsy, yet it caused very little accumulation of pleural fluid after intraperitoneal injection.

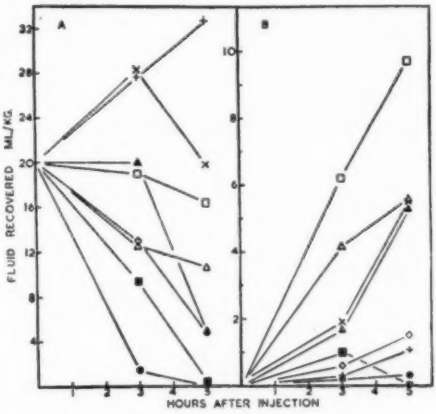

Fig. 1. Volumes of fluid recovered from peritoneal cavity, Fig. 1(A), and pleural cavity, Fig. 1(B), in rats after intraperitoneal injection of homologous plasma and of 7 different varieties of foreign plasma, 20 ml./kg.

Varieties of plasma: ●——● rat (homologous); ×——× bovine; +——+ human; □——□ rabbit; ■——■ guinea-pig; △——△ sheep; ▲——▲ dog; ◇——◇ horse.

In rabbits. Human plasma gave results similar to those in rats, but the experiments were complicated by gross capillary damage locally and by generalized intravascular haemolysis.

In guinea-pigs. Intravascular haemolysis did not occur after intraperitoneal injection of rabbit plasma, but, as Table 2 shows, there was evidence of exudation from the peritoneal capillaries as in the rat. *Post-mortem* examina-

TABLE 2.

Volumes of fluid recovered, in ml./kg., 5 hours after intraperitoneal injection in guinea-pigs of fresh rabbit plasma, 20 ml./kg. Mean results after guinea-pig plasma, 20 ml./kg., are included for comparison.

Type of plasma injected		Volume of fluid recovered	
		Peritoneal	Pleural
Rabbit		21·5	1·6
		21·1	0·5
		26·1	0·9
		19·4	3·7
		17·0	2·8
	Mean	21·6	1·9
Guinea-pig	Mean	10·3	0·6

tion showed the usual differences in appearance between animals which received the foreign plasma and those which received homologous plasma.

A local capillary reaction to foreign plasma in unsensitized animals is, therefore, not peculiar to the rat, nor is it peculiar to the peritoneal cavity, as the following experiments showed.

Intrapleural injection in rats.

Fresh homologous and foreign plasma, rabbit and human, was labelled with dye and injected. Groups of rats were killed at various intervals up to 24 hours after injection, and the volume of fluid remaining in the pleural cavity measured in each rat. The amount of labelled protein which had been absorbed was estimated from the concentration of dye in pooled samples of plasma from heart blood. The amount of undyed protein which had exuded from the capillaries into the pleural cavities was estimated from the amounts of protein and dye recovered in the pleural fluid, compared with the amounts of protein and dye injected. Due to the assumptions involved, such an estimate is valid only as a means of detecting a substantial increase of capillary permeability and of comparing gross differences in the degree of capillary damage.

Fig. 2 A, shows that after injection of foreign plasma there was a marked exudation into the pleural cavity, and Table 3 shows that this exudate was rich in protein. Once the period of rapid exudation was over (3 to 8 hours) the pleural fluid was absorbed at much the same rate after foreign as it was after homologous plasma. Fig. 2 B shows that labelled protein was absorbed steadily after injection of foreign plasma, despite concurrent exudation of undyed protein, although the rate of absorption of the label was somewhat slower than it was after homologous plasma.

The general picture after the intrapleural injection of foreign plasma was, then, the same as after intraperitoneal injection.

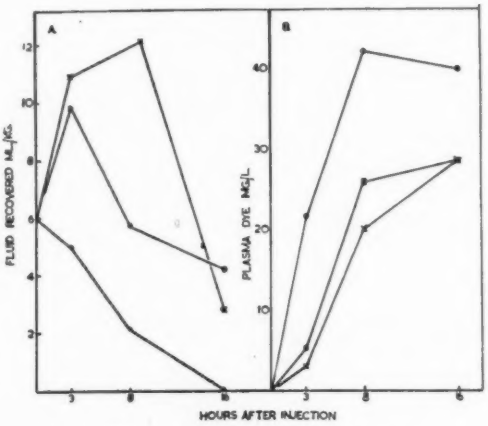

Fig. 2. Volumes of pleural fluid recovered, in ml./kg., Fig. 2(A), and concentrations of dye-protein in the circulation, in mg. dye/litre plasma; Fig. 2(B), after intrapleural injection in rats of foreign and homologous plasma, 6 ml./kg., containing T1824, 4 mg./kg. body weight.

Varieties of plasma: ●——● rat (homologous); ×——× human; ■——■ rabbit.

There were differences in detail. For example, the absorption of fluid was slower from pleural than from peritoneal cavity and there was an exudation of some protein even after homologous plasma was injected intrapleurally. These differences did not obscure the fact that foreign plasma caused a gross increase of permeability after intrapleural injection as well as after intraperitoneal injection in non-sensitized animals.

TABLE 3.

The volumes of fluid recovered, in ml/kg., and the estimated protein exudation, in mg./kg., after intrapleural injection in rats of foreign and homologous plasma, 6 ml./kg. containing T1824, 4 mg./kg. body weight. Mean values for groups of 3 to 9 rats.

Type of plasma injected	Hours after injection	No. of rats	Volume of fluid recovered	Protein exuded
Human	3	5	10·9	334
	8	6	12·2	248
	16	6	2·7	148
Rabbit	3	4	9·9	344
	8	7	5·8	113
	16	8	4·3	160
Rat	3	9	5·1	91
	8	9	2·0	41
	16	3	0	0

Intradermal injections.

An increase of capillary permeability as a result of the intradermal injection of a substance can be demonstrated if the circulating plasma proteins are previously labelled with T1824. The leakage of protein is then manifest as a blue staining of the tissues at the site of injection, if capillary damage is not extreme. With this well known technique, in rabbits and rats, a marked increase in capillary permeability was usually apparent after intradermal injection of foreign plasma or serum. Thus human plasma gave a marked reaction in 5/5 rats and rabbit plasma in 6/6 rats. In rabbits, human plasma or serum gave a marked reaction in 22/22 animals, rat plasma in 2/3, sheep and dog each in 2/2, guinea-pig plasma in 1/1 animals. An equivocal result was obtained with horse plasma 1/2 and rat plasma 1/3.

In summary, then, foreign plasma produced a marked increase in capillary permeability when injected intraperitoneally, intrapleurally and intradermally. In all, plasma from seven different species was used and the effects tested in three different species of laboratory animals, none of which had been subjected to immunization or sensitization. The range of the experiments performed is indicated in Table 4.

TABLE 4.

The foreign plasma reaction.

Donor species	No. of donor batches	Recipient species	No. of recipient animals	Injection site
Rabbit	5	Rat	37	Pleura
	16		134	Peritoneum
	2		6	Skin
Human	4		43	Pleura
	11		112	Peritoneum
	2		5	Skin
Horse	2		19	Peritoneum
Dog	2		15	Peritoneum
Sheep	2		14	Peritoneum
Cow	2		39	Peritoneum
Guinea-pig	1		13	Peritoneum
Human	1	Rabbit	9	Peritoneum
Human	10		22	Skin
Human	1		1	Conjunctiva
Sheep	1		3	Skin
Dog	1		2	Skin
Horse	1		2	Skin
Rat	2		3	Skin
Rabbit	1	Guinea-pig	5	Peritoneum

Duration of foreign plasma effect.

Inspection of curves showing the volumes of fluid recovered at various times after intraperitoneal or intrapleural injection of foreign plasma suggested that the period of rapid exudation was relatively brief. After this period, lasting between 3 and 8 hours, fluid was absorbed from peritoneal or pleural cavities as rapidly as if it were homologous plasma.

To demonstrate more clearly the duration of the foreign plasma effect, undyed human and rabbit plasma samples were injected intraperitoneally and intrapleurally in rats. The proteins of the circulating plasma in the rats were then labelled by injecting T1824 intravenously, 4 mg./kg., in aqueous solution one hour before the conclusion of the experiment. An estimate of the permeability of the capillaries during, for example, the fifth hour after injection of the foreign plasma was provided by injecting dye intravenously at the end of the fourth hour and measuring the amount of dye recovered in the pleural or peritoneal fluid at the end of the fifth hour. Some of the exuded dye-protein would be absorbed by lymphatics between injection of dye and removal of fluid, but this period was constant (viz. 1 hour) throughout the experiments. This source of error should therefore have been reasonably consistent. To assess the permeability of the capillaries when all the foreign plasma had been reabsorbed, for example during the 24th hour after intraperitoneal injection, homologous plasma, 20 ml./kg., was injected intraperitoneally immediately after the intravenous injection of dye.

The results are shown graphically in Fig. 3, the rate of exudation being expressed as mg. dye $\times 10^{-3}$/kg./hour. It can be seen that the rate of dye-protein exudation diminished rapidly shortly after intraperitoneal or intrapleural injection of human plasma. Eight hours after injection the rate of exudation was less than one-quarter the rate shortly after injection, and by 16 hours exudation was negligible. With rabbit plasma the exudation of dye-protein was less and the results of intrapleural injection were inconclusive, probably because the dye concentrations to be measured were of the order of 1–4 mg./litre. Five hours after intraperitoneal injection the exudation of labelled protein was very small, less than one-quarter the rate in the third hour after injection. These results indicate that although foreign plasma in unsensitized animals may markedly alter capillary permeability, or even their fragility, its effect is of limited duration.

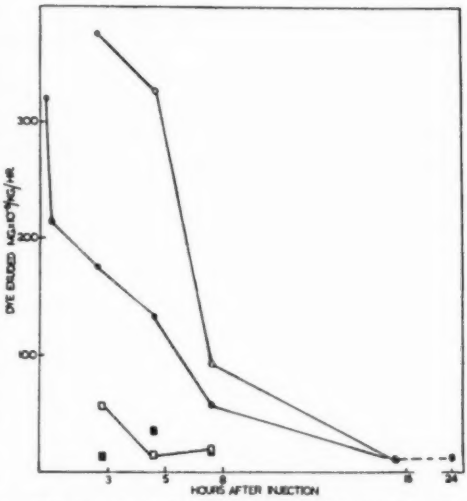

Fig. 3. Exudation of dye-labelled protein, as mg. dye $\times$ 10^{-3}/kg./hour, into the peritoneal and pleural cavities in rats after injection of undyed foreign and homologous plasma.

○——○ = human plasma intraperitoneally;
●——● = human plasma intrapleurally;
□—–□ = rabbit plasma intraperitoneally;
■–——■ = rabbit plasma intrapleurally.

DISCUSSION.

These experiments indicate that the effect of foreign plasma, increasing capillary permeability in unsensitized animals, is a general phenomenon. It has been demonstrated after intraperitoneal, intrapleural and intradermal injection in the rat, after intraperitoneal injection in the guinea-pig and after intraperitoneal and intradermal injection in the rabbit. There seems little doubt that a similar effect would occur with capillaries in regions other than those tested and in recipients other than the three species investigated. For example, Flexner and Amoss (1914) noted that horse serum, injected intraspinally in monkeys, produced an aseptic meningitis. Homologous serum caused similar but less pronounced changes.

The seven varieties of foreign plasma tested all produced a marked increase in capillary permeability compared with homologous plasma, but the degree of capillary damage appeared to vary, some varieties being consistently more

potent than others. These differences could not be ascribed to differences in nutrition, sex, or age in the recipient animals. Although pilot experiments showed that variability of response to intraperitoneal injections in rats was reduced by supervision of food and water intake during the previous 24 hours, the degree of exudation was not significantly affected. The evidence supports a real species difference in the foreign plasma effect. In this respect, it is interesting to refer to the work of Salter (1898) and Friendenthal and Lewandowsky (1899). These workers independently confirmed and amplified earlier observations (Guinard and Dumarest, 1897 a; Weiss, 1897; Creite, 1869), that foreign plasma had an almost immediate lethal effect when injected intravenously. With the plasma from some foreign species the lethal dose was much smaller than with others. The cause of death was not stated, and in some cases the lethal doses were so large that the recipient animals, usually rabbits, must have died from circulatory overloading. Again, the effect of cross-species haemagglutination cannot be excluded. It is of considerable interest, however, that these authors agreed that bovine serum was highly toxic while horse serum was slightly toxic. Human serum also appeared to be highly toxic (Guinard and Dumarest, 1897 b; Baylac, 1897). It will be recalled that human and bovine serum cause a marked increase in capillary permeability while horse serum has a much smaller effect. The early workers also described a decrease of intravenous lethal effect with aseptic storage of foreign plasma, and Courtice and Steinbeck (1951) have demonstrated a similar decrease in effect regarding peritoneal exudation. These resemblances may be fortuitous. It is unlikely that their further investigation would be productive until the factor or factors responsible for the local effect on capillary permeability have been identified. Some progress in this direction will be reported in the next paper (Lake, Simmonds and Steinbeck, 1953).

SUMMARY.

Foreign plasma, human and rabbit, produced an increased capillary permeability and exudation after intrapleural and intradermal injection in unsensitized rats as well as after intraperitoneal injection. The effect was not peculiar to the rat. It was demonstrated by intraperitoneal injection of rabbit plasma in the guinea-pig and of human plasma in the rabbit and by intradermal injection in the rabbit of plasma from a number of different species.

Dog, sheep and horse plasma, intraperitoneally in rats, had a similar effect to other foreign plasmas—bovine, rabbit and guinea-pig—but there appeared to be species differences in intensity of effect. With all foreign plasma tested so far except human, there has been an accumulation of fluid in the pleural cavity following intraperitoneal injection in rats.

The plasma of some foreign species, especially human, produced a massive exudation within the first few hours after intraperitoneal or intrapleural injection in the rat. The irritant effect, however, seemed to be brief, since the rate at which labelled protein escaped from damaged capillaries was greatly reduced within 8 hours, or less, after injection.

Acknowledgments. We wish to thank Dr. F. C. Courtice for his active interest and for helpful criticism. We should also like to thank Dr. R. J. Walsh, Director of the N.S.W. Red Cross Blood Transfusion Service, the members of his staff and the volunteer donors, for their co-operation in providing fresh human plasma.

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THE NATURE OF THE CAPILLARY DAMAGING FACTOR IN FOREIGN PLASMA

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The increase in capillary permeability produced by the injection of fresh foreign plasma in unsensitized animals did not occur after the plasma had been stored aseptically at 4° C. for 5 to 6 weeks (Courtice and Steinbeck, 1951). This suggested that the foreign plasma effect was due to the action of one or more of the labile constituents. Since homologous plasma had little or no effect on capillary permeability, it seemed likely that the foreign factor was capable of acting as one of the components of an antigen-antibody reaction. The object of this paper is to report progress in the identification of the active factor and to refer briefly to some experiments on the mechanism of its action.

It seemed probable that the effect resided in the protein or protein-bound constituents, but experiments were first carried out to confirm this by injecting human C.S.F. and ultrafiltrates of human plasma into rats. The effects of some purified foreign plasma fractions were then tested. Indirect evidence incriminated the unstable lipoprotein fraction and it was decided to test the effect of decrease and increase of this fraction in foreign plasma. The lipoprotein was disrupted or altered, *in vitro*, by ether treatment and increased, *in vivo*, by cholesterol feeding of rabbits. Regarding the mechanism, an effort has been made to uncover evidence of an antigen-antibody reaction.

METHODS.

The general methods and the dosage employed in intraperitoneal and intrapleural injections in rats and in intradermal injections in rabbits were the same as in previous investigations (Courtice and Steinbeck, 1951; Lake, Simmonds and Steinbeck, 1953). Dry heparin was used as an anticoagulant for injected plasma.

Ultrafiltration of plasma or serum. The method of Wilson and Holiday (1933) was used. Ultrafiltrates remained clear or gave a faintly opalescent ring when layered with salicyl sulphonic or trichloracetic acid.

Ether treatment of plasma or serum. In some cases the lipoproteins were disrupted by McFarlane's method (1942). In other cases similar ether treatment was carried out at 0°

[1] This work was carried out with the aid of a grant from the National Health and Medical Research Council, Canberra.

to 10° C. To determine whether the resultant decrease in the foreign plasma effect was due to the removal of lipoid soluble material or to alteration of the lipoprotein complex, the ether layer was removed and dissolved ether blown off without frothing, by a gentle stream of air or pure carbon dioxide, in some cases. In others, all the ether was blown off without removal of the ether layer containing lipoid soluble material. In one experiment, the ether soluble material was made up to the original volume of the plasma with saline, the ether blown off and the saline suspension injected.

Cholesterol feeding experiments in rabbits. Three male rabbits were given cholesterol, 2 gm. daily evaporated from ethereal solution on to cabbage leaves, with an *ad libitum* diet of rabbit cubes and water. The cholesterol content of the plasma before the experiment began was 69 mg./100 ml. After feeding for 11 weeks it was 1,070 mg./100 ml., and pooled heparinized plasma from ear vein blood was injected intraperitoneally in rats. At 12½ weeks, cholesterol was discontinued and pooled plasma again injected intraperitoneally in rats. At 14 weeks, the cholesterol concentration was 1,600 mg./100 ml. while at 16 weeks it was 550 mg./100 ml. and a final pooled sample was prepared and injected as before. Cholesterol estimations were performed by Dr. H. S. H. Wardlaw, using the Liebermann-Burckhardt reaction.

Removal of heterophile agglutinins from foreign serum. Human serum was brought against an equal volume of packed rat cells at 37° C. for 15 minutes. One washing was sufficient to adsorb agglutinins as determined by a simple tube agglutinin test and by the more sensitive Coombs test, using standard anti-human serum prepared by Commonwealth Serum Laboratories, Melbourne. Haemolysis did not result. As a control, the same human serum was brought against compatible human red cells and washed as before.

Precipitin test. The fluid recovered 3 hours after intraperitoneal injection of 0·9 p.c. NaCl solution in one group of rats, and of human plasma in another, was spun at 3,000 r.p.m. for 15 minutes and the supernatant poured off. The cellular layer was twice frozen to —23° C. and then thawed, to liberate antigens which might be present on the cells of the exudate. A small amount of fluid above the more solid material was obtained after these manipulations. This was layered in precipitin tubes, at a fixed dilution of 1: 10 in one instance and in varying dilutions in another, against varying concentrations of a different foreign plasma from that used in the initial intraperitoneal injection.

RESULTS.

Nature of the capillary-damaging substance.

Non-diffusibility. On intradermal injection in rabbits the protein-free moiety of human plasma produced no capillary irritation. Thus, ultrafiltrate of human plasma caused no exudation in 3/3 rabbits, in all of which the original plasma and the concentrated plasma above the collodion membrane gave strong positive reactions. Human C.S.F. gave negative results in 2/2 rabbits, both of which gave strongly positive reactions with human plasma. On intraperitoneal injection in rats similar results were obtained (Table 1, a). An ultrafiltrate of human plasma was rapidly absorbed without evidence of irritant effect, while the original plasma caused gross exudation and the ultrafilter-concentrated plasma even more. These experiments provided strong evidence that the capillary-damaging activity resided in the protein of foreign plasma or in material bound to the protein.

TABLE 1.

Fluid recovered, in ml/kg., 5 hours after intraperitoneal injection in rats of foreign plasma, ultrafiltrate, and serum, 20 ml/kg. (Averages for groups of 4–5 rats).

Type of fluid injected	Protein concentration gm./100 ml.	N.P.N. mg./100 ml.	Fluid recovered	
			Peritoneal	Pleural
1 (a)				
Human plasma (concentrated)	9·09	—	45·7	0·6
Human plasma	6·96	33·5	29·4	0·6
Ultrafiltrate	0·088	39·0	0·2	0·4
1 (b)				
Human plasma 1.*			41·2	1·4
Human serum 1.			36·3	8·1
Human plasma 2.			29·4	0·6
Human serum 2.			8·6	1·0
Rabbit plasma 1.			20·6	9·7
Rabbit serum 1.			25·4	18·4
Rabbit plasma 2.			13·3	10·7
Rabbit serum 2.			21·2	9·8

* The response to this sample of human plasma was significantly different from that with all other human plasma except the pathological samples reported in Table 4 (b).

Presence in serum as well as in plasma. Table 1 (b) shows that human and rabbit serum produced a peritoneal exudation in rats comparable with that after injection of plasma prepared from the same blood. After injection of serum the volume of peritoneal or of pleural fluid recovered was significantly different from that after plasma in individual experiments, but not consistently so. The foreign plasma effect, then, could not be ascribed to fibrinogen or other components of the clotting process.

The differences between heparinized plasma and serum in individual batches cannot be explained. Morrison (1946) found that the lipoprotein fractions, III_0 and IV_1, have specially high tendencies to occlusion in the clot as it forms. Since evidence presented below suggests that the foreign plasma activity is associated with the lipoproteins, Morrison's findings may account for some of the variability in serum activity, e.g. Human Plasma 2, Table 1 (b). Occlusion of a possible lipoprotein factor could not explain an apparently enhanced activity in serum, e.g. Rabbit Plasma 2. Heparin, which we used as an anticoagulant, has a complex effect on lipoproteins *in vivo* (Affinsen, Boyle and Brown, 1952), but there is little to suggest that it grossly affects lipoproteins *in vitro*. Whatever the explanation of the differences, both serum and plasma were alike in producing a marked "foreign plasma effect."

Presence in globulin, but not in albumin, fraction. To identify the capillary-damaging factor with one or more of the protein fractions, it was obviously desirable to test systematically a number of purified preparations by intraperitoneal, intrapleural and intradermal injection in several recipient species. How-

TABLE 2.

Results of intradermal injection in rabbits of foreign plasma protein fractions.

A positive result connotes a marked exudation of dye-labelled rabbit plasma protein from the dermal capillaries into 2/2 intradermal blebs of unâyed foreign plasma protein fraction in a given rabbit. Where all blebs were stained only light blue or where one of the duplicate or triplicate injections was only light stained the result is scored "positive (1)". All controls (rabbit plasma) were negative.

Material	Source	Protein concentration gm. p.c.	Positive	Negative
Bovine albumin	Crystalline	4·0		2
Human albumin	"Lilley"	4·25		1
		15·0		1
α globulin	M.R.C.	12·09	2	
		4·0		1
α globulin	"Lilley"	20·44	1	
		4·1	1	
α globulin	"Lilley"		1	1
α globulin	"Squibb"		2	
α globulin	(bovine)	15·0	(2)	
α globulin	Fraction II	10·0		2
β globulin	Fraction III_1	10·0	(2)	
β globulin	Fraction III_2	10·0	1	1

Samples denoted Fraction II, III_1 and III_2 were from the same human plasma pool, provided through the kindness of Dr. P. L. Bazeley.

ever, only very small volumes of concentrated fractions were available. It was decided, therefore, to test these preparations by intradermal injection in rabbits. The results are set out in Table 2. Pure plasma albumin, human and bovine, produced no capillary exudation. Similarly, Courtice and Steinbeck (1951) found that pure bovine albumin, 5 gm./100 ml. injected intraperitoneally, was absorbed at much the same rate as homologous plasma. Some of the foreign globulin preparations which we injected produced a definite exudation from skin capillaries. In no case was this effect as marked as with fresh human plasma in the same rabbits, even although globulin solutions of 10–20 gm./100 ml. concentration were injected. The diminished activity might be attributable to a storage effect, since all the preparations were more than two years old. The results of these experiments were, therefore, inconclusive. In conjunction with the results of intraperitoneal injection of pure bovine albumin they provide, perhaps, some indication that the effect on capillary permeability was associated with the foreign globulin fraction rather than with the foreign plasma albumin. Much more reliable evidence on this point was provided by changes in the foreign plasma effect associated with alterations in the lipoglobulin complex.

Evidence for association with lipoprotein fraction. The earlier observations of Courtice and Steinbeck (1951), that the foreign plasma effect was progressively lost with aseptic storage at 4° C., suggested that a factor in the lipoprotein fraction might be involved. The lipoproteins, which are chiefly lipo-

globulins, are among the most labile of the non-diffusible constituents of plasma. Indeed, there is macroscopic evidence of their breakdown with storage, since previously clear serum turns opalescent, or milky, and fatty material settles on the walls of the vessel. Before testing the effect of procedures specifically designed to disrupt the lipoglobulin complex, it seemed of interest to determine if the temperature of storage played an important part in the loss of the foreign plasma effect. Fig. 1 shows that this was so. The lower two curves demonstrate that the same human heparinized plasma lost its power to provoke a peritoneal exudation in rats more rapidly when kept at 37° C., than when kept at 4° C. The plasma was prepared aseptically, penicillin being added as an extra safeguard. It was stored in small sealed ampoules whose contents were tested for sterility by culture at the time of injection. The effect of the same plasma was also tested intrapleurally and intradermally. After storage for 14 days at 37° C. it was absorbed as rapidly as homologous plasma from the pleural cavity in rats. After storage for 41 days at 4° C. it still induced a considerable pleural exudate. The results of skin tests in rabbits were less consistent. Plasma which had lost nearly all effect demonstrable by intraperitoneal or intrapleural injection in rats still produced a variable exudation of dye-protein into intradermal wheals in rabbits. This exudation was not consistently less, in the same animals, than with more active plasma, stored at 4° C. The rabbits' own plasma had no effect intradermally in any instance. The skin test is not a quantitative procedure and these results merely suggest that intradermal injection demonstrates the presence of the capillary factor in amounts too small to affect peritoneal or pleural absorption. With intraperitoneal and intrapleural injection, then, there was evidence that storage at 37° C. accelerated the loss of the foreign plasma effect. The uppermost curve in Fig. 1 shows that extremely active human plasma, from a case of diabetic retinopathy, steadily lost its effect when stored even at —23° C.

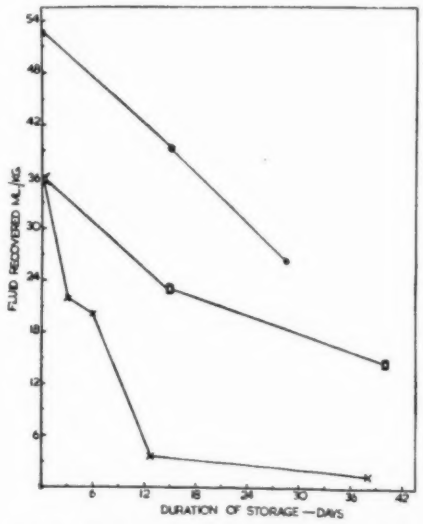

Fig. 1. The effect of duration of storage and of storage temperature on the exudation with human plasma, 20 ml./kg., injected intraperitoneally in rats.

●———● hyperactive plasma (diabetic retinopathy) stored at —23° C.
□———□ Normal plasma, stored at 4° C.
×———× Same plasma, stored at 37° C.

TABLE 3.

Effect of ether treatment on the amount of fluid recovered 5 hours after the intraperitoneal injection of foreign plasma or serum, 20 ml./kg., in rats. (Averages for groups of 4–6 rats).

Expt. No.	Material injected	Treatment	Fluid recovered ml./kg. Peritoneal	Pleural
1.	Human serum	Control	24·1	1·7
		Ether extracted at —25° C. (2)	6·0	1·6
		Frozen —25° C. and thaw (2)	24·3	1·1
2.	Human plasma	Control	27·5	2·7
		Ether extracted at 0° C. (1)	4·9	1·1
3.	Human serum	Control	28·0	4·6
		Ether extracted at —25° C. (2)	5·5	0·6
		Ether extracted at 10° C. (2)	2·5	1·2
4.	Rabbit serum	Control	22·5	7·9
		Ether extracted at 0° C. (1)	11·4	3·9
		Ether treated at 0° C. and ether blown off (1)	8·8	2·7
5.	Human serum	Control	19·4	4·6
		Ether extracted at —25° C. (1)	9·1	0·3
		Ether treated at —25° C. and ether blown off (1)	4·3	0·6
		Ether extractible material in saline	4·7	0·8

The figures in brackets indicate the number of ether treatments or extractions performed.

These results were consistent with the presence of a capillary damaging factor in the foreign lipoglobulins. To demonstrate this more conclusively it was decided to test the effect of ether treatment. The findings in these experiments are summarized in Table 3. Treatment with ether of serum frozen to —25° C., followed by thawing, breaks up the lipoprotein molecule, the lipoid being extracted into the ether (McFarlane, 1942). Half the total lipoid can be removed by one treatment but only exhaustive extraction will remove all of it. In Table 3, experiments 1, 3 and 5, it can be seen that one or two such treatments greatly diminished the peritoneal exudation produced in rats by fresh foreign serum. Ether treatment at 0 to 10° C. had a similar effect, experiments 2, 3 and 4. Over this temperature range, only small amounts of lipoid are extractable from fresh, non-lipemic serum, but it is very likely that the lipoproteins are partially denatured. There was some evidence that no generalized denaturation of serum proteins occurred, at least with McFarlane's procedure. Protein and non-protein nitrogens were not altered significantly in experiments 1, 2 and 3. In experiment 5 the hetero- and iso-haemagglutinin titres were shown to be unaltered and, in experiment 1, similar results were obtained for precipitin titres with rabbit anti-human serum. These five experiments strongly suggested, therefore, that the activity of foreign plasma or serum was mainly

connected with the lipoprotein fractions. The ether treated serum lost a great deal of its capillary damaging properties whether the ether soluble substances were subsequently removed or not.

TABLE 4.

Total volume of peritoneal and pleural fluid recovered, in ml./kg., after intraperitoneal injection in rats of normal and pathological samples of foreign plasma, 20 ml./kg. Each sample was injected into 3–5 rats and the values listed are the means for all samples x groups.

Type of plasma	Condition	Hours after injection	No. of samples	Mean volume of fluid collected	"p" for difference of means
4 (a)					
Rabbit	Cholesterol-fed	3	3	29·0	<0·02
Rabbit	Normal	3	9	23·4	
Rabbit	Cholesterol-fed	5	2	29·6	<0·01
Rabbit	Normal	5	4	23·3	
Rabbit	Cholesterol-fed	8	2	29·4	<0·05
Rabbit	Normal	8	2	14·6	
4 (b)					
Human	Malignant hypertension	5	1	40·5	<0·02
Human	Diabetic retinopathy	5	1	52·3	<0·001
Human	Normal	5	7	27·2	

Since decrease in the lipoprotein components of serum produced a decrease in the foreign plasma effect it was of interest to see if there was an enhanced effect with an increase in the lipoprotein fraction. High cholesterol feeding in rabbits produces an increase in the β globulins (Fishberg *et al.*, 1950) and the lipoproteins (Gofman *et al.*, 1950; Cook *et al.*, 1952) in the plasma. Table 4(a) shows that the plasma of cholesterolemic rabbits caused a significantly larger accumulation of peritoneal and pleural fluid than normal rabbit plasma. Again, the β globulin area of the electrophoretic pattern is increased in plasma from patients with malignant hypertension (Lewis and Page, 1947) and with diabetic retinopathy in the exudative phase (Lewis, Schneider and McCullagh, 1944). Table 4(b) shows that plasma from two such patients was significantly more active than normal human plasma, when injected intraperitoneally in rats. These results suggest that increase in lipoproteins or in the β globulin fraction may be associated with an increase in the foreign plasma effect.

Mechanism of foreign plasma effect.

Attempts to elucidate the mechanism by which foreign plasma causes an increase in capillary permeability have yielded inconclusive results, so far. Two methods of approach have been adopted. Attempts have been made to suppress the reaction by the use of antihistamine drugs and of A.C.T.H., which appear to

interfere with different types of mechanism producing capillary damage. We have also tried to adduce more direct evidence that the phenomenon is an immunological one.

TABLE 5.

Effect on the foreign plasma reaction in rats of premedication, with an antihistamine and with A.C.T.H., and of immunological manipulation of the foreign plasma. 20 ml./kg. body weight injected intraperitoneally. Mean values, in ml./kg., for fluid recovered in groups of 3–4 rats.

Plasma injected	Premedication of rats	Dose	Treatment of plasma	Volume of fluid recovered		Hours after injection
				Peritoneal	Pleural	
5 (a)						
Rabbit	Phenergan	6–12 mg./kg.	—	13·0	7·4	3
Rabbit	Nil	—	—	15·1	7·9	3
Rabbit	—	—	Phenergan added, 6 mg./kg.	16·6	3·8	3
Rabbit	—	—	Nil	14·1	4·6	3
Human	Phenergan	6 mg/kg.	—	26·9	1·5	3
Human	Nil	—	—	28·4	1·7	3
Rabbit	Phenergan	6 mg/kg.	Rabbit mixed with equal parts rat plasma	6·9	7·1	3
Rabbit	Nil	—	As above	9·9	6·4	3
Rat	Phenergan	6 mg/kg.	—	4·2	—	3
Human	A.C.T.H.	10 mg./rat	—	13·2	0·5	5
Human	Nil	—	—	27·3	0·2	5
5 (b)						
Human (serum)	—	—	Complement inactivated	1·2	—	5
Human (serum)	—	—	Control	16·7	4·5	5
Human (serum)	—	—	Minus haemagglutinins for for rat erythrocytes	20·6	4·2	5
Human (serum)	—	—	Control	23·2	5·5	5
Rabbit	—	—	Recovered after injection			
Rabbit	—	—	into dead rats	13·6	7·9	3
			Control	22·2	3·9	3

We are grateful to Mr. Buchanan, of May & Baker Pty. Ltd., for supplies of "phenergan".

Antihistamine drugs. There is evidence that the release of histamine is, at least partly, responsible for the increase in capillary permeability after physical or chemical injury and in certain immunological reactions, e.g. urticaria. With certain reservations (Gaddum, 1951), suppression of such an increased permeability by antihistamine drugs indicates that histamine plays a part. However, antihistamines did not have a consistent effect on the foreign plasma effect. Phenergan (promethazine) greatly diminished the skin reaction to foreign plasma. This occurred in 4/4 rabbits with human plasma and in rats with rabbit plasma 4/4 and human plasma 3/3. The dosage employed, 6–12 mg./kg. in rabbits and in rats, had a similar effect on injections of histamine, 0·02 mg. and 0·2 mg., as acid phosphate in 0·2 ml. saline, and of dilute HCl. Phenergan, however, did not affect the peritoneal exudation in rats with human or rabbit plasma, as Table 5(a) shows. Mixing of phenergan with the foreign plasma

before injection, or dilution of the foreign plasma with homologous plasma to decrease the capillary damage, did not uncover a protective effect. It seems unlikely, then, that "extrinsic" histamine (Dale, 1948) plays a part in the foreign plasma effect in the peritoneal cavity. Anthisan (neoantergan), a less powerful but allegedly more specific antihistamine, had irregular effects on the skin reaction.

A.C.T.H. Premedication with A.C.T.H. is said to reduce or suppress capillary damage due to hyaluronidase (Benditt, Schiller, Wong and Dorfman, 1950) and, immunologically, to suppress the Shwartzman phenomenon (Shwartzman, Schneierson and Soffer, 1950) and allergic reactions of the "delayed" type (Grob, Winkenwerder and Schoenrich, 1952). It does not suppress the effects of histamine (Grob *et al.*, 1952). Table 5(a) suggests that A.C.T.H. may diminish the foreign plasma effect, but further experiments are necessary.

Immunological experiments. The results of attempts at immunological manipulations of the foreign plasma before injection are summarized in Table 5(b). It can be seen that adsorption of haemagglutinins for rat red cells did not significantly affect the reaction. "Passaging" of the foreign plasma in recently dead rats appeared to reduce the activity, which suggests that it might be possible to demonstrate, by less equivocal methods, adsorption of the foreign plasma factor on to the fixed cells of rat tissues. We were unable to demonstrate a precipitin reaction between foreign plasma and possible antigens, liberated by deep-freezing and thawing, on the cells of peritoneal exudates in rats.

DISCUSSION.

In this and in previous papers, the increase in capillary permeability produced in unsensitized rats, rabbits and guinea-pigs by the injection of plasma of another species has been called the foreign plasma effect. This implies that homologous plasma caused little if any exudation of protein. Its innocuous nature was demonstrated by the virtual absence of dye-protein exudation when the circulating proteins were labelled with T1824 and undyed homologous plasma injected intraperitoneally and intradermally in rats, and intradermally in rabbits. There was some protein leakage after intrapleural injection in rats, but very much more in the case of foreign plasma (Lake, Simmonds and Steinbeck, 1953). The term "foreign plasma effect" also implies that the capillary injury is related to the immunologically foreign character of the plasma. This is, perhaps, less securely established, but it is difficult to reconcile our results with a purely pharmacological explanation. In this paper, gross differences in the volumes of fluid recovered at given times after the intraperitoneal or intrapleural injection of plasma have been regarded as indicating gross differences in the amount of protein exudation produced. This assumption is justified by the results of previous experiments in which the rate of exudation of labelled protein or the total amount of exuded protein recovered has also been measured (Courtice and Steinbeck, 1951; Lake *et al.*, 1953).

The foreign plasma effect appears to be associated with the proteins of plasma and, more particularly, with the lipoprotein components. The loss of effect with storage, more rapidly at 37° C. than at 4° C., is consistent with a factor of lipoprotein character. The lipoproteins of human plasma migrate with the α and β globulins. Changes in these fractions, attributable to the lipoglobulins, have been shown to occur with storage, more rapidly at room temperature than with refrigeration (Moore, Roberts, Costello and Schonberger, 1949). Ether extraction of serum by McFarlane's method greatly reduced the foreign plasma effect. McFarlane (1942), and subsequently Moore *et al.* (1949) found that the only change detectable electrophoretically after such an extraction was a decrease in the lipoglobulins. Finally, there was an indication (Table 4) that an increased foreign plasma effect was associated with increase in the lipoproteins. It is reasonable to suggest, therefore, that the foreign plasma "factor" is lipoprotein in character. It is difficult to say whether the intact lipoprotein molecules, the lipoid or the protein moieties are responsible for the effect. The foreign plasma lost its effect after ether treatment whether the lipoid was removed or not, and the ether soluble material, suspended in saline, was not irritant. Ether treatment at 0° C. decreased the effect, although only a little lipoid is split off in this case. These results, however, do not prove that the intact lipoprotein was responsible, and not the lipoid alone. Some lipoid can be extracted even at 0° C., and the physical state of the lipoid is changed by ether treatment, in the sense that it will not re-enter the aqueous phase. Similarly, with storage the changes include an increase in the amount of lipoid extractible with ether (Ada and Walsh, 1944), alterations in the lipoid of the β lipoprotein and in solubility of the α lipoprotein (Cohn, 1949). The results of our experiments do not indicate which members of the complex family of lipoprotein molecules may be implicated in the foreign plasma effect. It may be of some significance that human serum is the only animal serum which has been reported to contain considerable quantities of the high molecular weight species, β lipoglobulin (Edsall, 1947). When injected intraperitoneally in rats human plasma differs from the other foreign plasma studied so far in causing little leakage of fluid from the lymphatics into the pleural cavity during absorption (see Courtice and Steinbeck, 1951). Locally, however, it causes a gross exudation and petechial haemorrhages. Such procedures as cholesterol feeding in rabbits apparently cause a change in the distribution of the molecular species as well as an increase in the lipoproteins as a whole (Jones *et al.*, 1951).

Our experiments, so far, have thrown little light on the mechanism of the foreign plasma effect. It does not appear to be related to the phenomenon of cross-species haemagglutination, since the haemagglutinin titre was not affected by ether treatment while adsorption of haemagglutins for rat red cells from human serum did not diminish the effect intraperitoneally in rats. Since foreign plasma differs so markedly from homologous plasma it appears that an antigen-antibody reaction is responsible, but this has not yet been demonstrated. The

liberation of histamine does not appear to be an intermediate stage, at least in the peritoneal reaction. Feldberg and Schacter (1952) have shown that horse serum releases histamine from the isolated skin in unsensitized cats but not in dogs unless previously sensitized. The effect was lost after boiling the serum. This may be related to our observations that phenergan inhibits dermal exudation with foreign plasma in rabbits and rats, but a non-specific inhibition is possible. The loss of effect on heating serum at 61° C. for 7 minutes does not show that complement plays a part in the reaction, since the lipoglobulins are almost certainly affected by this procedure.

So far as can be determined, the effect of foreign plasma on unsensitized capillaries has received little attention in the literature. Isolated observations reported in the course of investigations of the Arthus Phenomenon (Nordmann, 1931) or of vascular reactions in anaphylaxis (Abell and Schenk, 1938) suggest that the intradermal effect is well recognized. Reference has been made in a previous paper to the interesting resemblance between the effect locally on capillaries and the immediate toxic action of foreign plasma given intravenously. Further points of resemblance may now be mentioned, in the light of experiments reported in the present paper. Davis and Eaton (1942, a and b) showed that bovine plasma, serum or serum globulin was highly toxic, intravenously in dogs, while bovine serum albumin was not. Horse serum or serum globulin was also toxic, although less so than bovine, while horse serum albumin was innocuous at the time of injection. Absorption of bovine serum haemagglutinins for dog erythrocytes did not affect the toxic properties. Since the pulmonary capillaries are the first exposed to foreign plasma after intravenous injection, it would be interesting to know if pulmonary oedema occurred. Davis and Eaton refer to severe dyspnoea before death and to the presenec of blood stained mucus in the bronchi at autopsy, but do not specifically mention pulmonary oedema.

SUMMARY.

The nature of the factor responsible for the foreign plasma effect, an increase of capillary permeability occurring on injection into unsensitized animals, has been investigated.

The factor was shown to be a protein or bound to plasma protein, since human C.S.F. and ultrafiltrates of human plasma had no irritant effect intradermally in rabbits or intraperitoneally in rats. Both plasma and serum contained the factor.

The activity appeared to be in the lipoprotein fraction, since interference with the lipoproteins, by treatment of plasma or serum with ether, greatly reduced the effect. The activity decreased with storage of plasma, more rapidly at 37° C. than at 4° C., and this was consistent with a lipoprotein factor. Finally, the effect was increased with plasma from cholesterol-fed rabbits and from two patients with malignant hypertension and diabetic retinopathy. In these conditions the lipoproteins are increased. There was no evidence that foreign plasma albumin played a part in the effect.

The mechanism by which foreign plasma increases capillary permeability is unknown. An antihistamine drug, phenergan, suppressed dermal but not peritoneal exudation. Evidence was obtained that A.C.T.H. may decrease the peritoneal exudation. No direct evidence of an antigen-antibody reaction was uncovered. It was established that foreign haemagglutinins for rat red cells played no part in the reaction in rats.

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THE ABSORPTION OF LABELLED ERYTHROCYTES FROM THE SUBARACHNOID SPACE IN RABBITS

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Forty-eight hours after blood was injected into the cisterna magna in rabbits, few red cells remained in the subarachnoid space. A considerable proportion disappeared during the first 24 hours (Simmonds, 1952). In these experiments few red cells appeared to be removed by such processes as clotting or enmeshing of cells in the arachnoid network, phagocytosis or haemolysis. This suggested that a considerable proportion of the injected erythrocytes passed, intact, out of the subarachnoid space. Blood was seen in the nasal submucosa and an appreciable, but small, amount was collected in the cervical lymph in anaesthetized cats kept in the head-down position. Virtually no cells seemed to pass through the spinal meninges. It was suggested that red cells passed, like protein molecules (Courtice and Simmonds, 1951), directly into the circulation, presumably through the arachnoid villi.

To determine the extent to which intact red cells could pass from the C.S.F. into the circulation, it was decided to label them, before cisternal injection in rabbits, with radioactive phosphate. The proportion of labelled cells absorbed could then be estimated from the radioactivity in samples of ear vein blood collected at various times after injection. Comparison of the absorption rates in rabbits with cervical lymphatics ligated and with the lymphatics intact should provide a measure of the rate of absorption of labelled cells directly into the circulation.

METHODS.

Labelling of erythrocytes with P^{32}. The method of Reeve and Veall (1949) was followed. About 4μC. of P^{32}, as phosphate, was added to 4 ml. of rabbit's heparinized blood, which was then rotated in a stoppered tube at 38° C. for 2 hours. At the end of this period about 50 p.c. of the radioactivity had passed into the red cells. The cells were washed with 8 ml. of ice cold 0·85 p.c. NaCl solution, three times, and resuspended in 1·6 ml. of plasma, free from radioactivity, from another rabbit. In most experiments the blood was injected into

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two rabbits, one of which was the donor of the corpuscles and the other the donor of the plasma. In the first ten experiments, slide tests showed no agglutination of the corpuscles by the added plasma. Isohaemagglutinins are not normally detectable in rabbit plasma (e.g. Snyder, 1924). After cisternal injection there was no systematic difference between homologous cells and the rabbit's own red cells in absorption rate for the first 5 hours. Similarly, Armin, Grant, Pels and Reeve (1952) found no significant difference between blood volumes in rabbits measured with homologous labelled red cells and those with autologous cells. In the present experiments, however, 16 hours after cisternal injection a much smaller proportion of homologous than of autologous cells was absorbed. The difference was highly significant ($p<0{\cdot}001$). Therefore, only data from animals injected with their own labelled red cells were used to calculate the absorption rates at 16 hr. There was no difference in the rate of disappearance of radioactivity in the first 16 hours after intravenous injection of homologous and of autologous cells. Thus the difference must have been associated with the mechanism of subarachnoid absorption, but no adequate explanation can be advanced at present.

Dose and method of injection. 0·5 ml. of labelled blood/kg. body weight was injected into the cisterna magna, after removing an approximately equal volume of C.S.F., by the method described by Courtice and Simmonds (1951).

Ligation of lymphatics. In such experiments both deep cervical lymph ducts were ligated in the neck near their entry into the great veins. Large tributaries from the superficial lymphatics, near the external jugular veins, were also ligated. The plasma proteins of the blood injected into the cisterna magna were labelled with the blue dye, T1824. Inspection at autopsy revealed no dyed lymphatics which had escaped ligation.

Measurement of radioactivity. Heparinized samples, 2 to 5 ml. of ear vein blood, were made up to 17 ml. with the phosphate buffer-citrate mixture recommended by Reeve and Veall (1949) and stored in the refrigerator. A sample of the radioactive blood used for injection was similarly treated, after appropriate dilution. On the following day, the radioactivity was measured after haemolysis with saponin in a liquid counting Geiger-Muller tube holding 17 ml. Estimates of radioactivity were based on 1,000 to 3,000 counts in ear vein blood samples and 5,000 to 10,000 counts in the case of the blood used for injection. Counting rates for ear vein blood samples were seldom less than twice the background and usually 3 to 5 times the background.

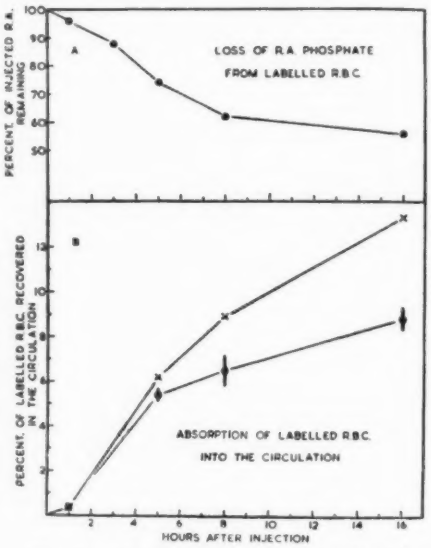
PERCENT. OF INJECTED R.A. REMAINING
A
LOSS OF R.A. PHOSPHATE FROM LABELLED R.B.C.
B
PERCENT. OF LABELLED R.B.C. RECOVERED IN THE CIRCULATION
ABSORPTION OF LABELLED R.B.C. INTO THE CIRCULATION
HOURS AFTER INJECTION

Fig. 1. Mean rate of loss of radioactive phosphate from labelled erythrocytes injected intravenously, 1(A), and mean rate of absorption of labelled cells from subarachnoid space into the circulation, 1(B).

●——● Mean percentage (± S.E.M.) of injected radioactivity present in circulation.

×——× Mean percentage of injected labelled erythrocytes present in circulation (see text).

RESULTS.

Rate of absorption of labelled cells.

In these experiments, 8 rabbits were used. The animals were anaesthetized for the cisternal injection, with a small dose of nembutal followed by open ether, and then allowed to recover. One to two hours later they were moving about normally in their cages. An intermediate blood sample of 2 ml. was taken from the ear vein 1 or 5 hours after injection and final samples of 5 ml. taken 5, 8 or 16 hours after injection in different rabbits. The lower curve in Fig. 1 B shows the amount of radioactive phosphate present in the circulation at various times after injection. A blood volume of 60 ml./kg. body weight has been assumed, in converting the measured radioactivity in counts/min./ml. into the percentage of injected counts which was present in the circulation. Measurement of the radioactivity in plasma and in whole blood showed that, in from 5 to 16 hours, 85 to 90 p.c. of the P^{32} in circulation was in the corpuscles. Thus the lower curve in Fig. 1 B shows that there was a steady absorption of intact labelled red cells into the circulation. However, the rate of absorption must have been greater than this curve suggests, since radioactive phosphate was being lost continually from the corpuscles after they were labelled, washed and injected. Fig. 1 A shows the average rate of loss of radioactivity from the corpuscles when labelled cells, prepared in the usual way, were injected intravenously in 8 rabbits. The mean rate of loss, about 5 p.c. per hour, agrees with that determined by Armin, Grant, Pels and Reęve (1952) in 29 rabbits in the first 35 minutes after injection. To estimate the percentage of labelled cells which had been absorbed at a given time after injection it was, therefore, necessary to correct the values shown in the lower curve, Fig. 1 B. The radioactivity in whole blood was reduced by 15 p.c. in each case, to allow for the radioactivity in the plasma. The resultant estimate of radioactivity in the corpuscles was then multiplied by a factor representing the radioactivity lost since the time of injection: for example, at 5 hours by 100/75, at 16 hours by 100/56. The upper curve, Fig. 1 B, shows the estimated rate of absorption of labelled cells when these corrections had been applied. This rate of absorption, about 1 p.c. per hour, would account for a considerable amount of the blood which disappeared from the C.S.F. during the first 16 hours after injection.

Pathways of absorption of labelled cells.

Experiments with labelled plasma proteins (Courtice and Simmonds, 1951) showed that, after cisternal injection, some protein molecules passed into the circulation by way of the cervical lymphatics while the majority followed a more direct route, presumably through arachnoid villi. If this were also true for red cells ligation of the lymphatics should not greatly affect the rate of absorption of labelled cells. Fig. 2 shows that this was so. The mean absorption rate was the same in rabbits with cervical lymphatics ligated as in rabbits with lympha-

tics intact, Fig. 2 A, when the animals were anaesthetized only for operation and the injection of blood. A similar comparison was made with rabbits anaesthetized throughout the experiment and kept in the left lateral position, head-down, at an angle of $22\frac{1}{2}°$. Fig. 2 B shows that, again, the rate of absorption was unaffected by lymphatic ligation. An analysis of variance of the data summarized in Fig. 2 showed that the absorption of labelled red cells was significantly slower in anaesthetized animals in the head-down position, whether the lymphatics were ligated or not. The relevant statistical findings are given in Table 1. These experiments, therefore, demonstrate that the majority of the labelled red cells passed into the circulation by some route other than the lymphatics, and that absorption was slower in anaesthetized animals in the head-down position.

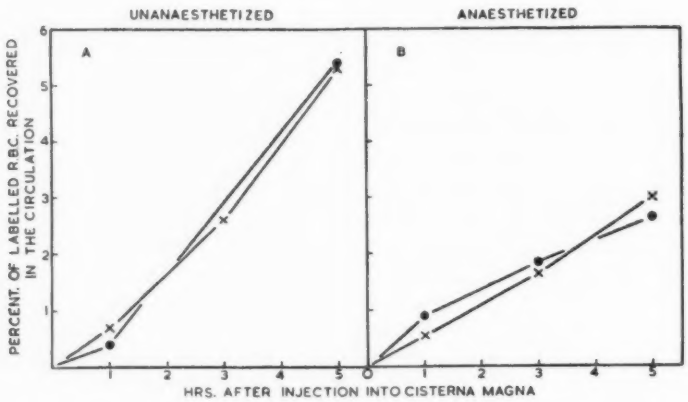

Fig. 2. Mean rate of absorption of labelled erythrocytes from subarachnoid space in rabbits with cervical lymphatics ligated or intact; 2(A), unanaesthetized, normal posture; 2(B), anaesthetized, head down at $22\frac{1}{2}°$.

●——● Lymphatics intact. ×——× Lymphatics ligated.

TABLE 1.

Analysis of variance of percentage absorption of labelled red cells into the circulation.

Item	Sum of squares	Degrees of freedom	Mean square	Variance ratio	Probability
Treatments*	21·62	1	21·62	25·4	<0·001
Subgroups†	0·56	1	0·56	1·5	~0·9
Subgroups × Treatments	0·04	1	0·04	21·3	~0·2
Error	10·2	12	0·85	—	—

* Anaesthesia plus head-down posture or unanaesthetized, normal posture. † Lymphatics ligated or lymphatics intact.

TABLE 2.

Mean concentration of cells remaining in C.S.F. 5 hours after injection of blood, 0·5 ml./kg. body weight. Concentrations are expressed as a percentage of the concentration in injected blood.

Experiment	No. of animals	Mean ± S.E.M.
Labelled cells, anaesthetized, head down	7	15·3 ± 3·6
Labelled cells, unanaesthetized, normal posture	7	22·3 ± 2·2
Above, corrected (see text)		26·8 ± 2·7
Unlabelled cells, unanaesthetized, normal posture	6	22·5 ± 4·1

In a previous paper it was shown that the mean concentration of red cells in the C.S.F. declined more rapidly, with unanaesthetized animals, in the head-down position at an angle of 45° than in a normal posture. Table 2 shows that the mean concentration of the P^{32} label fell to a somewhat lower level with anaesthetized animals head-down at 22½° than it did in normal unanaesthetized animals. The difference was much less than with animals at 45° and was not statistically significant. The dose of injected material, 0·5 ml./kg., was the same as in previous experiments. It can be seen from Table 2 that, with normal unanaesthetized animals, the decline in C.S.F. concentration of P^{32} was the same as the decline in erythrocyte counts with unlabelled blood. The agreement was still good when account was taken of the loss of radioactivity from labelled cells and of the amount of free radioactivity in the C.S.F., as explained in regard to Fig. 1 B. In anaesthetized animals in the head-down position absorption of labelled cells was slower than normal although the concentration of cells in the C.S.F. declined possibly more rapidly than normal. Changes in the rate of disappearance of red cells from the C.S.F. during the first 5 hours after injection seemingly afford little indication of alteration in the rate at which intact cells ultimately return to the circulation. The very rapid disappearance of unlabelled cells in unanaesthetized animals head-down at 45° therefore indicated, as stated previously, that cells were being removed from the C.S.F. more rapidly by speedier passage through the meninges and, possibly, by lodging in parts of the subarachnoid space inaccessible to C.S.F. sampling. It did not necessarily mean that they were returning to the circulation more rapidly.

DISCUSSION.

P^{32} is, in some respects, an unsatisfactory label for erythrocytes, since the cells subsequently exchange the radioactive phosphate fairly rapidly. It possesses the advantage that the animal's own cells can be labelled. As mentioned in the section on methods, this appears to be important when the fate of the

injected cells is studied during fairly long periods after injection. This discrepancy between the absorption of homologous and autologous cells needs further investigation. Pending this, the P^{32} method, despite the empirical corrections employed, seems preferable to the use of cells permanently labelled with radioactive iron, from a donor animal. The free radioactive phosphate which entered the C.S.F. from labelled cells and was then absorbed did not introduce a serious error, by entering unlabelled cells in the circulation, since radioactive phosphate is rapidly taken up by such tissues as liver and bone. When the usual dose of radioactivity was injected cisternally as phosphate in separated plasma, less than 1 p.c. of the dose appeared in the circulating corpuscles. Moreover, the circulating radioactivity, measured at 1, 3 and 5 hours, remained quite steady. When labelled cells were injected cisternally, less than 10 p.c. of the radioactivity was present in the cell free layer of C.S.F. samples taken up to 16 hours later. Entry of free label into circulating unlabelled cells must, therefore, have been negligible.

The experiments which have been described support the suggestion (Simmonds, 1952), that intact red cells, despite their size, pass at an appreciable rate out of the subarachnoid space through the meninges. It may be of interest to point out that protein molecules appeared to pass into the circulation about three times as rapidly as red blood cells, although it would be difficult, and might be misleading, to attempt a close comparison. About 20 p.c. of injected dye-protein (Courtice and Simmonds, 1951) and about 7 p.c. of labelled red cells were present in the circulation 5 hours after cisternal injection of the same volumes of plasma and of whole blood. Similarly, the subarachnoid space was almost free from plasma in 1 day and from blood 2 to 3 days after injection. The passage of intact red cells in considerable numbers through the meninges and into the circulation may be of some significance pathologically. Jackson (1949) has shown, in dogs, that the breakdown products of haemoglobin are much more irritant to the meninges than intact autogenous red cells. If the escape of free red cells, not entangled in clot, is comparable in other species to that in the rabbit, the amount of blood requiring phagocytosis and breakdown after subarachnoid haemorrhage would be reduced. This should decrease such sequelae as irritation and fibrosis of the meninges.

Regarding the pathways by which erythrocytes are absorbed, the majority of labelled cells appeared to by-pass the lymphatics. Ligation of the main deep and superficial cervical lymph ducts interrupts most of the lymphatic drainage of the subarachnoid space (Courtice and Simmonds, 1951). This procedure did not measurably affect the rate of passage of labelled erythrocytes into the circulation. It seems likely that the arachnoid villi constitute the non-lymphatic pathway for absorption of both labelled protein and labelled cells, but this would be difficult to prove. The rate of absorption, relative to the cerebral blood flow, is too slow to permit a satisfactory demonstration of a venous sinus-

arterial difference in concentration of the label. Erythrocytes can be seen in the villi after subarachnoid haemorrhage, or the injection of blood, and in selected histological sections may appear to be passing into the sinus. It seems unlikely that radioautographs could show more than this.

Further investigation of the factors affecting the disappearance of erythrocytes from the C.S.F. and their reappearance in the circulation will be necessary. Such a study should include lymphatic cannulation in unanaesthetized animals. For example, labelled cells appeared more slowly in the circulation with anaesthetized animals in the head-down position, whether the lymphatics were ligated or not. Yet, more red cells were recovered in lymph from anaesthetized cats in the head-down position (Simmonds, 1952). However, massage of the tissues or passive flexion of the head had to be employed to maintain a flow of lymph. In the experiments with labelled cells this was not done and there was little or no spontaneous neck movement to propel the lymph towards the blood stream.

SUMMARY.

The rate and pathways of absorption of erythrocytes from the subarachnoid space in rabbits was investigated by labelling homologous cells or the rabbits' own cells with radioactive phosphate and resuspending them in homologous plasma, free from radioactivity, before injection.

After cisternal replacement injection of 0·5 ml. labelled blood/kg. body weight, the labelled cells were steadily absorbed into the circulation at a rate of about 1 p.c. of the injected dose per hour, for the first 16 hours. At 16 hours, but not at 5 hours, significantly less homologous cells had been absorbed than rabbits' own cells.

Ligation of the main cervical lymphatics did not affect the rate of absorption. The majority of the labelled cells must have been absorbed by some non-lymphatic pathway, presumably the arachnoid villi. Absorption was significantly slower in anaesthetized animals kept in the head-down position.

Acknowledgments. I should like to thank Dr. F. C. Courtice for his interest and for helpful criticism. I should also like to thank Professor W. P. Rogers, Department of Zoology, Adelaide University, for advice on the use of P^{32}; Mr. Gill, McMaster Laboratory, C.S.I.R.O., Sydney, and Professor P. M. de Burgh, Department of Bacteriology, University of Sydney, for permission to use liquid counting G–M tubes in their departments; and Dr. P. J. Korner for advice and assistance in statistical procedures.

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THE ENDOCRINE IMBALANCE INDUCED IN FEMALE RATS BY AUTOTRANSPLANTATION OF THE OVARY INTO THE TAIL

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Some of the effects produced in female rats by autoplastic transplantation of ovaries into the tip of the tail have been described by Hernandez (1943). Such ovarian grafts secreted appreciable amounts of androgens, as shown by the stimulation of clitoris and rudimentary prostate, and also enough oestrogen to maintain continuous oestrus for many weeks. Histological examination of ovaries transplanted into the tail revealed that they consist mainly of follicles, the source of the oestrogen secreted, and of luteinized theca cells, the producers of androgens. Corpora lutea were absent. The pituitaries of these rats were enlarged. Hernandez assumed that this was due to the action of oestrogens, the daily production of which he considered to be equivalent to that of daily injections of 1 and in some instances of 5 R.U.

In this paper we shall deal only with the effects of the gynecogenic action of tail grafted ovaries and with the changes in the cytology and the gonadotrophin content of the pituitaries of rats bearing such autotransplants. The females of our strain are unsuitable for the study of androgenic activity. The evidence submitted by Hernandez and the findings of many workers, recently reviewed by Parkes (1950), do not leave any doubt as to the ability of ovarian grafts to secrete androgens.

MATERIALS AND METHODS.

The animals used were females of the Wistar strain. At the age of 21–31 days the ovaries were extirpated and one or both transplanted into a pouch produced by removal of the last vertebrae of the tail. The wound was closed with a single suture. The operation was successful in most instances. The material presented in this paper is derived from 40 rats which showed prolonged periods of persistent oestrus up to the date when the animals were sacrificed. Vaginal smears were taken daily until continuous oestrus became established, and later on at weekly intervals. The animals received ordinary stock diet and their weight was recorded once a week. From all animals sacrificed material was taken for histological study of the graft, vagina, uterus and mammary gland. Of the pituitaries 23 were used for histological purposes after previous weighing, and 17 were assayed for their gonadotrophin content. The pituitary of the donor was triturated in a small mortar and the material

suspended in 1 ml. of saline. The recipient, a 21-day-old intact female, was injected twice daily for three consecutive days and sacrificed 96 hours after the first injection.

Three pairs of females were joined in parabiosis using the method of Jacobsohn (1948). The ovaries of one partner were removed and those of the other were autotransplanted into the tip of the tail.

The steroid hormones were administered by subcutaneous injections, the dose given being contained in 0·1 to 0·2 ml. of a mixture consisting of 2 parts of olive oil and 1 part of castor oil.

RESULTS.

The vagina opened 8–14 days after the transplantation of the ovary into the tip of the tail. The interval between operation and opening of the vagina was the same for animals operated on when 21 days old as for those 31 days of age. The earliest date on which opening of the vagina was observed was the 30th day of life, i.e. considerably earlier than is the rule for normal females of our strain. In the majority of these the vagina opens at the age of 42–46 days. Of 64 normal animals examined, only one showed this phenomenon when 38 days old; the others 2–12 days later. Therefore transplantation of ovaries into the tail can hasten the appearance of the first oestrus provided the operation is undertaken during the first half of the 4th week of life.

We have compared the growth rate of tail-grafted females with those of spayed animals and of females bearing an ovarian graft in axilla or lung. There was no difference between the weights of animals having transplants in the tail and showing continuous oestrus, and of rats with lung or subcutaneous grafts and showing oestrus cycles. Spayed rats increased in weight more rapidly and reached higher levels. To give one example: a litter of five females was operated when 30 days old; three of these were used for transplantation of the ovaries into the tail and the other two were gonadectomized. The former weighed 228, 182, and 211 gm. when five months old, the spayed litter mates 260 and 261 gm. respectively. The weight curve of the females bearing ovarian grafts suggests that the transplants secreted enough oestrogen to prevent the additional gain in weight characteristic for spayed females, but did not secrete growth-inhibitory amounts of oestrogen.

Vaginal smears.

In some animals oestrus was found to be present continuously from the day of the first smear onwards, i.e. from the day when the vagina opened. During the whole period of observation cornified epithelial cells predominated in the smears with only occasionally a few leucocytes present. In other rats it took 2–3 weeks after the opening of the vagina until the condition of persistent oestrus became established. During this period smears showing the typical picture of oestrus alternated with those of anoestrus in an irregular fashion. The survival of the grafts as judged by the appearance of the vaginal smears varied from 6 months to at least one year, when the last animals bearing a func-

tional graft were killed. After six or more months of continuous oestrus a number of grafts degenerated as indicated by the appearance of increasing numbers of leucocytes in the smear. The post-mortem examination of such animals revealed the secondary sex organs to be atrophic. As already stated, the material dealt with in the paper consists only of animals which had been in continuous oestrus up to the date on which they were sacrificed.

The grafts.

The outstanding feature of the grafts was their size. None reached the weight of an ovary of a normal adult rat or of an ovary transplanted successfully into lung or axilla. The majority remained stationary during the length of the experiment, i.e. at the size of an infantile gland. Hernandez (1943) has given a well documented description of the histology of ovarian grafts transplanted into the tail, and our findings are in conformity with his; but in addition to paraffin embedded material we have also studied frozen sections. Amount and distribution of sudanophilic material in tail-grafted ovaries varied according to the age of the transplant. A frozen section stained with Oil Red O and Haematoxylin is depicted in Fig. 1. It was obtained from an animal which had been in persistent oestrus for 18 days. The theca surrounding the large follicles contains a fair amount of lipoid but most of the sudanophilic material is found in the interstitial tissue, a picture resembling that of an infantile ovary stimulated by F.S.H. After longer periods of persistent oestrus the amount of Oil Red O stainable material in the ovary diminished and its distribution became patchy and irregular. Figs. 2 and 3 show grafts of 4 and 6 months' standing. The former transplant contained large agglomerations of theca cells besides follicles, the latter consisting of anovular follicles separated by strands of theca cells. In both, the theca contained relatively little Oil Red O stainable material, but in some areas the mesenchymal cells were loaded with coarse droplets of lipoid. The findings suggest that little steroid material was stored in the older grafts.

The histology of vagina, uterus and mammary gland.

The first experimental animal was sacrificed 15 days after transplantation, i.e. after 5 days of uninterrupted oestrus. The vagina was lined by stratified squamous epithelium showing heavy keratinization. This appearance of the vagina did not alter to any marked degree during the whole length of the experiment and the thickness of its epithelial layer did not increase progressively. Fig. 4 shows a typical example taken from a rat which had been in continuous oestrus for 217 days.

Only in two rats, killed 5 and 7 days after continuous oestrus had become established, the uterus resembled that of a normal rat in oestrus. Later on the amount of fluid present in the lumen became negligible, and no fluid was pre-

sent in rats which had been in oestrus for four or more weeks. The histological appearance of the uterus changed also with the duration of oestrus. In the beginning, as seen in Fig. 5, the lumen was fairly wide and the epithelium lining it tall cylindrical with elongated nuclei. The mucosa was cellular and the number of glands present was normal. Whereas the lining epithelium changed little, the number of glands and the cellularity decreased progressively and the tunica became increasingly fibrotic. After two or more months of continuous oestrus, foci of squamous metaplasia appeared in the lining as well as in the glandular epithelium (Figs. 6 and 7). This change from cylindrical to squamous epithelium was present in only a few of the animals sacrificed before the sixth month, but of the seven rats who had been in oestrus for 217-355 days, four showed this phenomenon to a remarkable degree.

Less pronounced were the changes in the mammary glands of rats sacrificed during the first months of the experiment. Definite signs of stimulation were found only in the breasts of three rats which had been in continuous oestrus for 10–12 months. Macroscopically, cysts containing milky fluid were recognizable and the mammary glands occupied a larger area than normally. Histologically, the ducts were found to be dilated, filled with eosinophilic material and their epithelium showed obvious signs of secretory activity (Fig. 8). The hyperplasia was restricted to the ducts and there was hardly any alveolar development.

The histological appearance of vagina, uterus and mammary gland is that of stimulation by oestrogen. It was most pronounced in the vagina, well recognizable in the uterus and occasionally present in breast glands of rats in which the graft remained functionally active for 10–12 months. There was

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EXPLANATION OF PLATES.

Fig. 1. Distribution of lipoid in ovarian graft of rat with persistent oestrus for 18 days (Oil Red O and Haematoxylin). × 23.

Fig. 2. Distribution of lipoid in ovarian graft of rat with persistent oestrus for 4 months. (Oil Red O preparation). Patchy distribution of lipoid. × 39.

Fig. 3. Distribution of lipoid in ovarian graft of rat with persistent oestrus for 6 months. (Oil Red O preparation). Some lipoid in theca surrounding follicles; in one area accumulation of Oil Red O stainable material. × 39.

Fig. 4. Vagina (persistent oestrus for 7 months). H. & E. × 114.

Fig. 5. Endometrium (persistent oestrus for 5 days). H. & E. × 114.

Fig. 6. Focus of squamous metaplasia in epithelium lining uterine cavity (persistent oestrus for 10 months). H. & E. × 339.

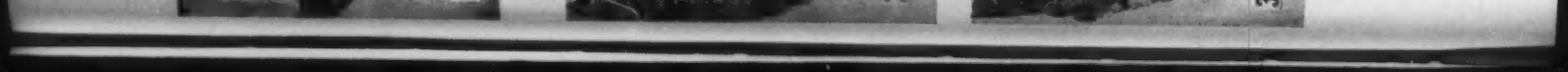
Fig. 7. Squamous metaplasia of glandular epithelium of the uterus (persistent oestrus for 7 months). H. & E. × 72.

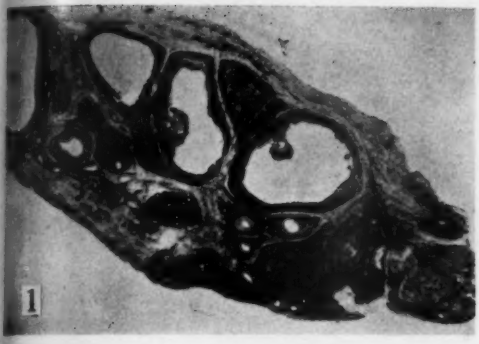
Fig. 8. Cystic hyperplasia of mammary ducts (persistent oestrus for 10 months). H. & E. × 72.

Fig. 9. Proliferating glandular epithelium of uterus (persistent oestrus for 324 days). H. & E. × 339.

Fig. 10. Pituitary of same rat as in Fig. 8. The lighter areas are due to an increase in the gonadotrophs. Papanicolaou. × 39.

Fig. 11. Detail from Fig. 10 showing a number of typical gonadotrophs lining a capillary. The acidophils are black. Papanicolaou. × 600.

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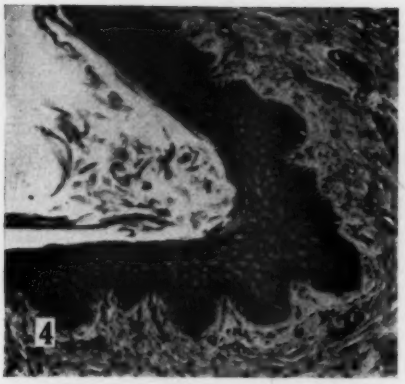
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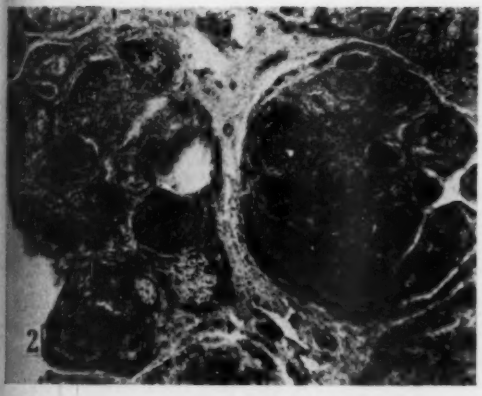
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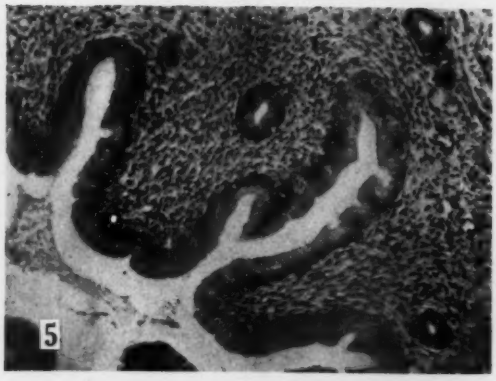
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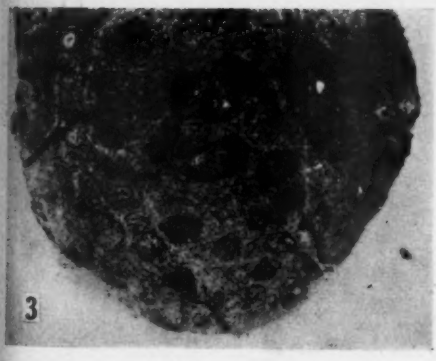
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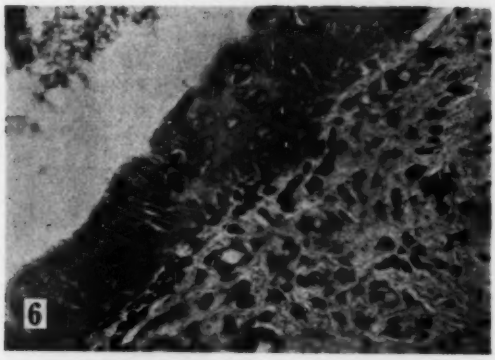
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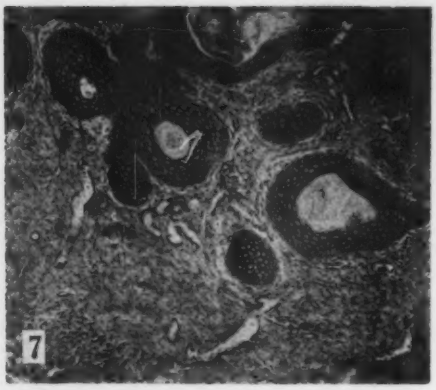
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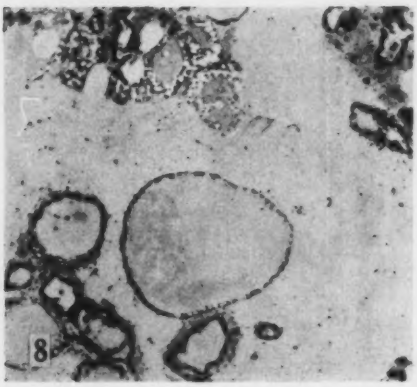
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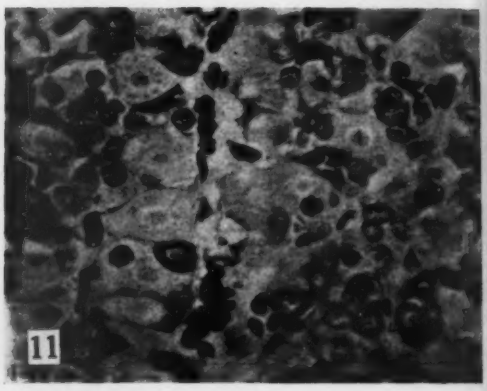
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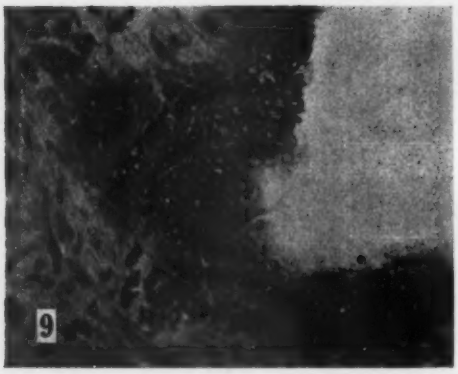
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no histological evidence for the action of other steroid hormones, such as progesterone or testosterone, with one possible exception. This rat had been in oestrus for 324 days. At autopsy the uterus appeared well stimulated and histologically it differed from the uteri of the other long term oestrus rats by the great number of endometrial glands, some of which were dilated. This was the only instance where a condition approaching cystic hyperplasia of the endometrium was present. Of special interest was the marked tendency of the glandular epithelium for proliferation. Fig. 9 shows the epithelium of a cystic gland growing into the stroma. The epithelium lining the uterus was cuboid and the cellularity of the mucosa unusually great. It is difficult to state with certainty which factors were operative in this case. The breast glands of the animal resembled more those of a male than of a female rat, and it is therefore possible that the histological changes in the uterus were the result of a combined oestrogen-androgen action rather than of oestrogen alone.

The pituitary.

At post-mortem examination the majority of the pituitaries appeared enlarged. The glands of twenty-three tail-grafted rats were weighed (Table 1) and in twenty-one instances were found to exceed the normal value, which averages 4 mg. per 100 gm. body weight for the intact virgin rat of our colony. Some were even larger than the pituitaries of spayed animals whose glands rarely surpass 6 mg. per 100 gm. body weight.

TABLE 1.

Body and pituitary weights of rats with persistent oestrus.

Days of oestrus	Body wt. gm.	Pit. wt. mg.	Body wt. mg./100 gm.
5	85	5·2	6·1
6	92	4·7	5·1
15	141	9·2	6·5
17	136	8·5	6·3
22	114	7·0	6·1
26	130	8·5	6·5
31	146	7·1	4·9
34	155	10·1	6·5
37	131	9·0	6·9
51	174	12·9	7·4
52	198	12·0	6·1
59	202	11·8	5·8
60	169	9·6	5·7
64	197	15·7	8·0
79	209	14·4	6·9
115	208	14·0	6·7
121	240	14·2	5·9
147	238	12·2	5·1
151	253	16·9	6·7
175	254	14·5	5·7
194	220	9·8	4·4
195	255	10·4	4·1
356	161	8·6	5·3

Histologically, the main feature of the pituitaries of the tail-grafted rats was an increase in the number of those basophils which Purves and Griesbach (1951a) named gonadotrophs. Without exception these cells were more numerous than in the pituitaries of intact virgins, and this increase was especially striking as rats in oestrus normally have rather few gonadotrophs. In a few cases the increase in gonadotrophs was moderate and these glands resembled the pituitary of a normal male rat; however, in the majority it was very pronounced and could be detected under low power magnification, as depicted in Fig. 10, where the light areas correspond to regions in which basophils predominate. Fig. 11 depicts the typical appearance of the gonadotrophs as seen in the pituitaries of tail-grafted rats. The cells are large, generally well granulated and have a Golgi body of fair size. They are especially numerous in the neighbourhood of blood vessels. Some vacuolated gonadotrophs, usually named castration cells, were found in the pituitaries of animals which had been in oestrus for 115 or more days, but they never became as numerous as in the glands of animals gonadectomized 3 or more months previously. For instance, in the case of a rat which had been in continuous oestrus for 356 days, they were rare. In the pituitaries of tail-grafted rats which had been in oestrus for shorter periods, the distribution of the gonadotrophs was more or less limited to the so-called sex zone (Purves and Griesbach, 1951a), but after several months these cells spread over large areas of the gland. The percentage of basophils stainable with Gomori's fuchsin-aldehyde stain always remained small, additional evidence that the overwhelming majority of the basophils were in fact gonadotrophs and not thyrotrophs (Purves and Griesbach, 1951b). The acidophils were normal as to numbers, size and stainability, except in the animals sacrificed shortly after continuous oestrus had become established, when they appeared to be more numerous. Only in one instance, in a rat sacrificed after 37 days of persistent oestrus, we found abnormally large and partially degranulated acidophils with a large Golgi apparatus as seen in pituitaries of rats after the administration of unphysiologically high amounts of oestrogen. Gonadotrophs, however, were still present.

These cytological findings are in good agreement with the results obtained by the assay of 17 pituitaries for gonadotrophic activity. In all instances these pituitaries were found to increase considerably the weight of the ovaries of the immature intact rat. In Table 2 the results of two typical experiments are given. In assay A the donors were four litter mates, operated on when 30 days old. In rat 1 the right ovary was removed and the other grafted into the tip of the tail; in rat 2 both ovaries were transplanted into the same site whereas rats 3 and 4 were gonadectomized. The animals were sacrificed 38 days later; the period of continuous oestrus lasted in the case of rat 1 for 24, and in the case of rat 2 for 28 days. The recipients belonged to one litter and were 21 days old when injections were commenced. As seen in Table 2 the increase in

ovarian weight obtained with the pituitaries of the spayed rats was slightly higher than with the pituitaries of the tail-grafted animals. Corpora lutea were induced by the extracts from rats 1, 3 and 4. Six other pituitaries of tail-grafted rats assayed 7–29 days after continuous oestrus had become established failed to induce corpora lutea whereas glands obtained from spayed animals and assayed two or more weeks after gonadectomy always induced multiple corpora lutea. However, all the seven pituitaries obtained from rats which had been in continuous oestrus for 6–10 months were rich in L.H. as well as in F.S.H. In Table 2 the results of an assay (B) are included in which the gonadotrophic activity of the pituitaries of two rats bearing functional grafts for 10 months was compared with that of pituitaries of two animals ovariectomized 5 months previously. Here again the increase in weight of the ovaries was of a similar order of magnitude and corpora lutea were found in all 4 recipients. These examples illustrate the remarkable and persistent increase in gonadotrophic potency of the pituitaries of tail-grafted rats.

The findings obtained in sex organs and pituitaries of the three tail-grafted rats joined in parabiosis to an ovariectomized litter mate were so similar with those described above that it seems unnecessary to recapitulate them. The only point we wish to stress is that the graft remained just as small as in the "single" rats. The transplants did not show the increase in size, nor were they of the appearance of ovaries of normal females joined in parabiosis to gonadectomized litter mates (Zeckwer, 1944).

TABLE 2.

Assays of pituitaries of tail-grafted female rats.

	Rat No.	Donor	Recipient ovaries mg.	Corpora lutea
Assay A.	1	Tail-grafted 37 days previously	62·1	present
	2	Tail-grafted 37 days previously	70·5	absent
	3	Spayed 37 days previously	77·6	present
	4	Spayed 37 days previously	80·7	present
Assay B.	1	Tail-grafted 10 months previously	62·0	present
	2	Tail-grafted 10 months previously	95·0	present
	3	Spayed 5 months previously	87·6	present
	4	Spayed 5 months previously	95·1	present
Controls (4)	—	(Saline only)	13–15	absent

The effects of various steroid hormones on the vaginal smear.

As the histological study of the ovarian grafts and of the secondary sex organs gave no evidence for the secretion of progesterone, it seemed of interest to test which dose of injected progesterone might interrupt the continuous oestrus. Progesterone in doses of 1 mg. or higher induced regularly a vaginal

smear in which leucocytes predominated. This change took place during the second day after the injection. It persisted for 5 days when 5 mg. had been administered, and for shorter periods with smaller amounts (Table 3). A dose of 0·7 mg. of progesterone induced a dioestrus smear in one of three animals, in the other two only a few leucocytes appeared among the still prevailing epithelial cells. Everett (1943) found that a single injection of 1 mg. of progesterone interrupted the persistent oestrus which occurs spontaneously in the D.A. strain of rats. The amount of progesterone needed to change the vaginal smear of tail-grafted rats was of the same order of magnitude, although the endocrine imbalance discussed in this paper varies in many respects from the syndrome described by Everett.

TABLE 3.

Effect of progesterone and testosterone proprionate on persistent oestrus.

	Dose mg.	No. of rats	Period of dioestrus, days
Progesterone	5	3	4–5
	2·5	5	2
	1·25	4	2
	1·0	5	1–2
	0·7*	3	1
Testosterone propionate	1	4	5–6
	0·5	9	4–5
	0·2	4	2–3
	0·1*	3	1

* 1 of 3 rats showed a vaginal smear characteristic for dioestrus.

Pregnenolone in doses of 10 mg. interrupted for 2 days the continuous oestrus of two of the four animals injected. During the same period the other two had a vaginal smear which consisted of a mixture of cornified cells and leucocytes. Testosterone propionate invariably interrupted the persistent oestrus when more than 0·1 mg. was given. Lower doses (0·05–0·1 mg.) produced irregular effects. After the injection of 0·5 mg., cornified cells were absent from the smear for at least five days. Androsterone in amounts up to 1·5 mg. was ineffective. Lack of this steroid prevented the assay of higher dosages.

DISCUSSION.

The reaction of vagina, uterus and mammary gland to the oestrogen secreted by the tail graft was a graded one. Whereas the vagina soon showed obvious signs of stimulation, evidence for stimulation in the mammary glands appeared late. After many weeks of persistent oestrus, cystic hyperplasia of the ducts was found in only a few animals. The squamous metaplasia of the

epithelium of the uterus as well as the cystic hyperplasia of the mammary gland are in our opinion due to an unopposed action of oestrogens rather than of hyperoestrinism. Pyometria were never seen, additional evidence that relatively small amounts of gynecogenic hormones were secreted. The females of our Wistar strain are very prone to develop pyometria when under the influence of elevated amounts of oestrogens. We have seen pyometria occur in animals bearing a single pellet consisting of 9·5 mg. of cholesterol and 0·5 mg. of stilboestrol. The cytology of the pituitary as well as the bio-assays are proof that the amounts of oestrogen secreted by the grafts were not large enough to re-establish the pituitary in its normal condition. As shown by Salhanick, Hisaw and Zarrow (1952) large amounts of oestrogen are necessary to reduce the gonadotrophin content of the castrate's pituitary to normal values in the absence of progesterone. The ovarian graft situated in the tail does not form corpora lutea, and there is no evidence that progesterone is formed by ovarian grafts transplanted into this site. These findings support the view that the endocrine imbalance induced by autotransplantation of ovaries into the tip of the tail is that of an ovarian deficiency, at least as far as its gynecogenic activity is concerned. The deficiency is characterized by oestrogen secretion which is constantly low and by the inability of the graft to form corpora lutea. This prevents cyclic variations in oestrogen secretion which, as pointed out recently by Barahona, Bruzzone and Lipschutz (1950), play an important rôle in the maintenance of the normal ovarian-pituitary relationship.

Hernandez (1943) expressed the view that persistent oestrus after ovarian transplantation into the tail is indirectly due to an increased androgenic activity of the ovary, the immediate effect of the latter being the suppression of the release of the luteinizing hormone by the hypophysis. The results obtained in parabiotic rats do not support this view. We prefer a different interpretation of the imbalance described in this paper. Many ovarian grafts independently of the site of transplantation lose earlier or later the ability to transform follicles into corpora lutea. When this happens, persistent oestrus results and at the same time the gonadotrophs of the pituitary increase in numbers and so-called castration cells appear. This stage precedes the degeneration of the graft (Pfeiffer, 1936). As far as the gynecogenic activity of tail grafts is concerned, the only difference between them and grafts implanted in the proximity of the jugular vein, or into the axilla, is that ovaries transplanted into the tail never form corpora lutea. Interference with the blood supply by a temporary ligature of the vessels entering the ovary (Fels, 1944) leads to an endocrine imbalance characterized by periods of prolonged oestrus—that is, failure to form corpora lutea—and the appearance of castration cells in the pituitary. We are therefore inclined to assume that in the case of the tail grafts the ovarian deficiency might be due to an inadequate blood supply. Such grafts grow extremely slowly and even when exposed, as in the parabiosis experiment, to the

high gonadotrophin content derived from the pituitary of the gonadectomized partner, they remain small, and the usual response to gonadotrophic stimulation, hyperaemia and growth, is lacking. This interpretation is strengthened by results from animals in which the entire thyroid gland was transplanted into the tip of the tail (unpublished results). Such rats showed a picture of nearly complete thyroidectomy. Their pituitaries contained many typical thyroidectomy cells and only a few weakly granulated acidophils were present.

Increased androgenic secretion of ovarian grafts under the influence of gonadotrophins has been described by many authors and not infrequently under conditions where corpora lutea were absent. There seems to exist an antagonism between androgen and progesterone secretion by the ovary. For instance, failure to form corpora lutea together with the appearance of signs of masculinization are the characteristic features of cystic hyperplasia of the ovary, a condition occurring in several species. Selye and Collip (1933) induced continuous oestrus in rats 6–21 days old with anterior-pituitary-like (A.P.L.) extracts, and found the ovaries to contain follicles in which a hyperplastic luteinized theca surrounded the granulosa which enclosed an ovum. Bradbury and Gaensbauer (1939) observed that besides continuous oestrus such treatment led to the appearance of signs of masculinization. Selye's A.P.L. preparations injected into older animals produced maturation of follicles and corpus luteum formation but "the infantile rat responds to gonadotrophic treatment in a bisexual manner by producing effective amounts of both oestrogenic and androgenic substances."

The reaction of the infantile ovary stimulated by A.P.L. is remarkably similar to that of the tail-grafted ovary. We are therefore inclined to assume that the androgenic secretion of these grafts may be due to an elevated gonadotrophin secretion of the pituitary. As early as 1938, Lipschutz pointed out the similarity of sexual disturbances after ovarian transplantation and after operative reduction of ovarian tissue (ovarian fragmentation). He found that the pituitaries of his experimental animals showed a gonadotrophic activity which reached that of castrates, although signs of "hyperoestrinism" such as cystic hyperplasia were present in the uterus. He found also in some guinea-pigs signs of masculinization appearing after these operative interventions.

To summarize, we consider the endocrine imbalance described in this paper to be the result of the following sequence of events: the ovary transplanted into the tip of the tail does not succeed in establishing an adequate blood supply. An ovarian deficiency results to which the pituitary reacts with a hyperplasia of the gonadotrophs.

SUMMARY.

Autotransplantation of ovaries into the tip of the tail induced continuous oestrus in rats.

The appearance of vagina, uterus and mammary gland of animals bearing functional tail grafts for periods up to one year has been described and interpreted as being due to unopposed oestrogenic stimulation.

The pituitaries of such rats contained an increased number of those basophils which secrete gonadotrophins. When assayed such pituitaries were found to be rich in F.S.H. in all instances and to contain also elevated amounts of L.H. when oestrus had persisted for prolonged periods.

The endocrine imbalance existing in rats bearing ovarian grafts in the tail is considered to be the result of an ovarian deficiency, characterized by an inability to form corpora lutea, by a persistent low secretion of oestrogen and by the production of appreciable amounts of androgen.

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THE PRODUCTION AND ABSORPTION OF VOLATILE FATTY ACIDS IN THE INTESTINE OF THE GUINEA-PIG

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From the point of view of their alimentary systems, herbivora may conveniently be placed in one of two main groups: (a) the ruminants and (b) those animals possessing a large caecum.

The physiology of the ruminant digestive apparatus has been intensively studied. The food, largely cellulose-containing material, is retained in the rumen for a considerable period of time. Here bacterial fermentation converts a proportion of the cellulose to fatty acids, which are then absorbed either directly through the mucous membrane lining the rumen or subsequently in their passage down the small intestine. Very little study has been made of the alimentary tracts of those herbivora possessing a simple stomach and large caecum. However, as they thrive on a diet consisting largely of cellulose, it is inferred that fermentive processes occur in their caeca similar to those taking place in the rumen. In this case absorption of the products of fermentation could only occur through the caecal or colonic mucous membrane.

The caecum of the guinea-pig is a large blind viscus with apertures of small intestine and colon close to each other at one end. In the adult it is about 10 cm. long and contains from 30 to 40 ml. of a homogeneous, light brown, viscous material. Even under deep anaesthesia, the caecal wall is continually contracting, thus ensuring a thorough mixing of the contents. After thorough mastication followed by salivary, gastric and intestinal digestion, food enters the caecum so that absorption of some of the digested food constituents may be assumed to have taken place prior to its arrival there.

In this investigation we have studied the duration of stay of food material in the caecum, the quantity and identity of volatile fatty acids present in caecal contents, the *in vitro* production of fatty acids from cellulose inoculated with caecal material and the ability of the various parts of the intestinal canal of the guinea-pig to absorb the sodium salts of acetic, propionic, butyric and valeric acids.

[1] This work was done with the aid of a grant from the Commonwealth Research Fund for Universities.

METHODS.

The length of time material was retained in the caecum was investigated radiologically. Barium sulphate was fed to 6 guinea-pigs by stomach tube and the animals X-rayed at frequent intervals throughout the subsequent 48 hours or longer. In order to keep them still, animals were lightly anaesthetized with ether immediately before radiography. The animals were allowed free access to food and water throughout the experiment.

The fatty acid content of caecal material from each of three guinea-pigs was determined by thoroughly mixing 1 gm. of the material with 10 ml. distilled water and submitting 1 ml. of the mixture to acidification with oxalic acid, steam distillation and titration with barium hydroxide using phenolphthalein as indicator. The distillate was brought quickly to the boil a few minutes before titration in order to get rid of CO_2, a blank was treated in the same way and appropriate correction made.

The volatile fatty acids in the distillate were identified by paper chromatography using the method of Reid and Lederer (1951). Standards were always run on the same sheet of paper so that identification by Rf values was unnecessary.

For investigation of the *in vitro* formation of volatile fatty acids a filter paper mush suspended in a phosphate buffer of pH 6·5 containing ½ p.c. urea as a source of nitrogen was inoculated with about 15 gm. of caecal material and thoroughly mixed, thus producing about 60 gm. of a homogeneous brei. 10 gm. of the brei were put into each of two test tubes which were stoppered with cotton wool and 10 gm. into each of two Thunberg tubes, which were then evacuated with a vacuum pump; 10 gm. were used for determining the initial volatile fatty acid content of the brei. The four tubes were incubated for 40 hours at 39° C. and on removal their volatile fatty acid content determined as described above.

For investigating the absorption of short chain fatty acids sodium pentobarbital anaesthesia was administered intraperitoneally.

The segment of intestine being studied was cut at either end, washed through with physiological saline until the washings were quite clear, then firmly ligated at both ends. The solution of carboxylic acid (as the sodium salt, pH 7) was introduced from a burette directly through a fine hypodermic needle inserted through the wall of the gut until the segment was just filled without overdistention. After two hours the intestinal segments were removed, opened and washed thoroughly with distilled water, the washings were made to volume and the volatile fatty acid content determined as before. The quantity absorbed was determined by subtracting the amount recovered from that introduced.

RESULTS.

In the six animals studied radiologically barium sulphate reached the caecum within one to two hours and immediately became distributed throughout the whole length of the organ. It could still be detected in the organ of the first guinea-pig 32 hours later but not at 48 hours; in the second pig, barium sulphate was still present at 73 hours but not after 78 hours; in the third pig at 30 hours but not at 55 hours. In the other three pigs it was still present in the last rays taken at 32 hours, 47 hours and 48 hours. Barium sulphate also appeared in the colon at the same time as it first appeared in the caecum, indicating that a certain proportion of the foodstuff escapes caecal fermentation.

The passage of barium sulphate through the alimentary canal of a guinea-pig is shown in the accompanying plate, illustrating the presence of barium in the caecum within 1¼ hours of feeding and still there at 32 hours. The 49-hour picture shows no barium.

The amount of volatile fatty acids present in 1 gm. of caecal contents of each of three guinea-pigs was found to be 53, 64 and 54 micro-moles. Chromatographic separation showed a consistently large spot of acetic, a smaller spot corresponding to propionic and a trace of butyric acid.

When cellulose was incubated with caecal material in the urea-containing phosphate buffer the following results were obtained:

Initial volatile fatty acid content	275 micro-moles.
Volatile fatty acid content after incubation of tubes exposed to air	690 and 550 micro-moles.
Volatile fatty acid content of Thunberg tubes after inoculation	690 and 960 micro-moles.

This indicates a considerable production of volatile fatty acid.

TABLE 1.

Absorption in mg. of fatty acids during 2 hours following their introduction into segments of the alimentary canal of the guinea-pig.

	Small intestine		Caecum		Colon	
	Introduced	Difference*	Introduced	Difference*	Introduced	Difference*
Sodium acetate 1·25 p.c.	44	—23	258	—212	37	—31
			228	—128	35	—30
Sodium propionate 2 p.c.	91	—76	1620	—620	66	—55
	78	—76	1710	—590	100	—93
Sodium butyrate 1 p.c.	48	—45	166	—136	54	—34
	32	—29	273	—243	41	—38
Sodium valerate 2 p.c.	73	—61	267	—207	87	—83
	120	—88	305	—222	90	—67

* Difference between amount introduced and that recovered.

The results of the study of the absorption of carboxylic acids are shown in the table. It can be seen that considerable absorption has taken place from small intestine, caecum and colon, that the caecum is obviously capable of absorption of large amounts of sodium salts of the fatty acids and the rates of absorption from small intestine and colon are approximately the same.

Plate showing the passage of barium sulphate through alimentary canal of a guinea-pig. Twenty minutes after feeding barium sulphate, the shadow can be seen in stomach and first part of small intestine. In 1½ hours a small amount of barium can be seen within the caecum. The two-hour photograph shows barium distributed diffusely throughout the caecum and at 2½ hours it appears in the colon. Within 5 hours dense shadows can be seen in caecum, colon and rectum and become less intense in the 24 and 32-hour pictures. At 49 hours no barium remains in the alimentary canal.

DISCUSSION.

It appears that the guinea-pig retains a certain proportion of ingested material in its caecum for a period of about 48 hours. The fact that barium appears in the colon at the same time as it becomes distributed throughout the caecum indicates that some foodstuff is not retained in the caecum, but excreted without fermentative digestion. Some of that which remains in the caecum undergoes fermentation there with the formation of short chain carboxylic acids similar to those formed in the rumen of the sheep. In the ruminant a considerable proportion of these volatile fatty acids is absorbed directly through the wall of the rumen and what escapes can be absorbed in the small intestine. In the guinea-pig most of these acids appear to be absorbed directly through the caecal wall and those which miss caecal absorption are able to be absorbed from the colon. Certainly both colon and caecum have the capacity of readily absorbing short chain volatile fatty acids.

SUMMARY.

Radiological investigation shows the retention of barium sulphate within the caecum of the guinea-pig for about 48 hours.

Analysis of the caecal contents of three guinea-pigs gave a concentration of about 50 to 60 micro-moles of volatile fatty acid (mainly acetate) per gm.

Volatile fatty acids have been produced *in vitro* from cellulose inoculated with caecal material from a guinea-pig.

The ready absorption of volatile fatty acids (as the sodium salt) from small intestine, caecum and colon has been demonstrated.

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